2014

A GENOMIC AND ELECTROPHYSIOLOGICAL STUDY OF PHOTORECEPTION IN THE EYELESS CNIDARIAN HYDRA VULGARIS

Stephanie L. Guertin
University of Rhode Island, stephanie.guertin@gmail.com

A GENOMIC AND ELECTROPHYSIOLOGICAL STUDY OF PHOTORECEPTION

IN THE EYELESS CNIDARIAN HYDRA VULGARIS.

BY

STEPHANIE L. GUERTIN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

INTERDISCIPLINARY NEUROSCIENCES

UNIVERSITY OF RHODE ISLAND

2014

DOCTOR OF PHILOSOPHY DISSERTATION

OF

STEPHANIE L. GUERTIN

APPROVED:

Thesis Committee:

Major Professor Gabriele Kass-Simon

Steven Q. Irvine

Linda A Hufnagel

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2014

ABSTRACT

Hydra are tubular coelenterates with two germ layers, the endoderm and ectoderm. A ring of five to eight tentacles surround an oral pore, and the animal attaches to the substrate via adhesion of the peduncle at the opposite end. They possess a complete ectodermal nerve net, with nerve fibers running within the ectoderm throughout the body and along the length of the tentacles (Hufnagel 1976.) Hydra has two nerve rings. One nerve ring surrounds the mouth and is thought to coordinate mouth opening. A second ring, located at the base of the tentacles, coordinates movements of the body, tentacles, mouth, and nematocysts in response to chemical, photic, and tactile stimuli. The functional unit of the tentacle effector systems is the battery cell complex. The battery cell complex consists of a large epithelial cell called a battery cell and its associated complement of neurons and nematocytes. These battery cell complexes link to each other via interdigitating neuronal processes and myonemes. They have been shown to respond to diverse chemical and mechanical stimuli. When separated from the battery cell, the nematocyst is still able to respond to mechanical and photic stimuli, though not chemical cues.

Hydra have been known to be photosensitive since the 1800's, and have been shown to demonstrate a preference for some colors over others. They are sensitive to light at the base of the animal. Exposure to light causes contractions of the endodermal musculature and extensions of the body column. Light exposure also changes the frequency of both ectodermal contraction pulses and endodermal rhythmic pulses. Further, I have found in my current electrophysiological experiments that hydra's ablated tentacles show some of the same differences in behavior across

wavelengths.

The Pax family of genes is found across taxa, from cnidarians all the way to humans. These highly conserved genes code for a transcription factor instrumental in the formation of the eye, to such a degree that it leads to the production of eyes where none should be. In fruit flies, exogenous expression of the *Pax6* protein product causes the production of eyes on the legs or antennae. Box jellies like *Cladonema,* members of another cnidarian group, express a version of the Pax genes that also creates ectopic eyes in *Drosophila.* Nine mammalian Pax genes have been identified, in four subgroups; most of the Pax family is involved in the development of the nervous system, in particular those sections dealing with optical input. The relationships between the Pax systems in more evolved organisms and those in more basal organisms have also been sought. A pair of Pax families, named *PaxA* and *PaxB,* has been found in both sea nettles and hydra. In addition, the protein products of the hydra *PaxA* gene were found to bind to a site for *Pax5/6* products, which means that the genes produce a very similar protein at all evolutionary levels. This similarity indicates that the Pax gene family has been involved in vision for a very long time. Because hydra are known to be basal to the eumetazoans, adding hydra to the list of Pax-expressing species, coupled with the hydra's simple nerve net, allows us to examine the roots of vision and color sensitivity in its most primitive form.

In this study of *PaxB* in hydra it was expressed during head regeneration and development, in the cell types expected to become neurons and nematocytes. Most particularly, the expression in the nematocytes is of interest, as it spatially couples the presence of *PaxB* to the response to light as demonstrated by Plachetzki and others.

The electrophysiological results reported here add further support to this, with the greatest response to light found in the large tentacle contraction pulses seen in ablated tentacles. These tentacles are rich with nematocytes and battery cells, further spatially linking light reception to these cell types.

ACKNOWLEDGEMENTS

I would like to dedicate this work to the people who have helped me get here, most notably my parents, Nancy and Phil Guertin. Your unflagging support and steadfast faith have made this possible; without you, I'd be nowhere. To Jon, for your belief that I would make it, and for making me believe it too, my eternal gratitude and love. To my friends, especially Clarissa, and Jethro, for listening to me vent, for endless proofreading, and for encouragement, my great thanks.

This work would not have been possible without the knowledge, guidance, and support of my major advisor, Gabriele Kass-Simon. Despite everything, she persisted – and persuaded me to keep on. She taught me so much, more than this dissertation can begin to show.

I would also like to thank my committee members, Steven Irvine and Linda Hufnagel. They accepted me into their labs, taught me invaluable techniques, and guided the direction of my research with endless patience and support. Many thanks also to Walt Besio and Brad Seibel for their advice and mentorship, and to James Smith for serving as my defense chair.

I would also like to thank my past and current labmates, coworkers, and friends, including Jarren Kay, Mike Sipala, Al Nyack, Vandana Nandivada, Bailey Munro, Steve Steinmetz, Bianca Lauro, Michelle Gonsalves, and Ryan Rettinger. Sometimes, what's most needed is to talk it out with someone to really see where the data is leading you.

Science increases our power in proportion as it lowers our pride.

Claude Bernard

PREFACE

This dissertation is presented in manuscript format in accordance with the guidelines set forth by the Graduate School of the University of Rhode Island. Each chapter is written to stand alone as a separate research question while simultaneously contributing to the greater body of knowledge about hydra neurophysiology and the evolution and development of the photic response in cnidarians specifically and early-evolving organisms generally. Chapter 2 is in preparation for submission to Development, Genes and Evolution. Chapters 3 and 4 are currently in preparation for submission to Comparative Biochemistry and Physiology A: Physiology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

FIGURE PAGE

CHAPTER 4

FIGURE PAGE

CHAPTER 1

Introduction and Literature Review

Genomic evidence has shown that the freshwater polyp *Hydra vulgaris* is closely related to the ancestor of all eumetazoans - including sea anemones, insects, birds, mammals, and humans (Putnam et al. 2007). This close relationship, coupled with the simple, yet complete, nerve net found in the species, makes hydra an ideal experimental organism for examining a number of questions dealing with the early evolution of nervous transmission and sensing (Kass-Simon and Scappaticci 2002).

Photoreception is one of the oldest and most widespread sensory systems, with examples going all the way from bacteria and algae to humans. The structures, proteins, and pathways involved vary widely, but the end result is the same: a system that plays a role in feeding, reproduction, circadian rhythm, and many other vital processes. A significant amount of evidence indicates that cnidarians are the earliest-evolving organisms to produce distinct eyes, defined as specialized organs with a photosensory function (Arendt 2003). See Figure 1 for a tree of cnidarian evolutionary relationships. Further, they even form several different types of photosensitive structures, sometimes even more than one type in a single species, representing the development of eyes in miniature. Both the medusoid and polypoid species have photosensitive abilities. The medusoids in particular have been heavily studied, particularly the box jellies like *Cladonema californicum*, with their large, varied, and easily identifiable photosensitive structures (Sun 2001, Bentlage 2009). By contrast, though a number of polyp species, like *Hydra,* demonstrate a marked photosensitivity, the genes, pathways, and cell types responsible remain largely unclear. Although there is one polyp species known to have pigment-spot ocelli at the base of its tentacles, further analysis shows that this species, *Stylocoronella riedli*, is

actually a sessile medusa (Bentlage 2009). Nevertheless, it may be used as a reference point for investigating the photoreception of true polyps. Some further assumptions may be made about their likely methods and form based on other cnidarian visual equipment, as will be outlined below.

Cnidarians have many different types of photosensors (Martin 2002, Coates 2003, Kozmik 2008). The simplest are eyespots, patches of pigmented epithelium associated with ciliated sensory cells. The precise shape and size of the receptor cells varies with species. The next in the line of ascending complexity is the pigment-cup ocellus. In these, the sensory cells are contained within a cup formed of pigmented epithelial cells. The cup has the advantage of directing the light input, making it possible for the animal to determine directionality of the input. It also concentrates the light to some degree, though not to the same resolution as lensed eyes. A similar type to this in both complexity and structure is the slit eye found in cubomedusae (Coates 2003). These types of photosensors are only dimly image-forming at best, most likely capable only of distinguishing a shadowy pattern of lights and darks.

Further up the complexity series are the complex eyes, which come in both large and small types (Martin 2002). These complex eyes, also referred to as the camera type, have a cornea, a cellular lens, a retina with pigmented cells, and, in some cases, a vitreous space. These eyes are quite morphologically similar to the camera type eyes of cephalopods and vertebrates, and may be assumed to have fairly similar image-forming properties. The lens is cellular, but the cells appear to form a partial syncytium, lacking cells walls, discrete nuclei, and internal structure. The lens is continuous with the retina, with no space between as is found in vertebrate eyes. In

some species, the lens does appear to secrete a closely associated capsule that is sometimes referred to as the vitreous body.

Many species of cnidarian also display extraocular photosensitivity, with neurons, epithelial cells, and myonemes playing a role in the light response. Of particular interest is neuronal photosensitivity, in which the neurons act as the receptors, the effectors, and, in those species with at least semi-complete nervous networks or rings, the integrators of the photoresponse. Some genomic evidence has suggested this as the earliest type of photosensitivity, one which remains extant in many species up to the present.

Despite the differences in structure of the photosensitive organs, the photoreceptive cells themselves are fairly uniform. All cnidarian photoreceptors thus far found are of the ciliary type, with a 9+2 bundling arrangement in the microtubules, the same construction as vertebrate photoreceptors (Kozmik 2008, Shubin 2009). This serves to reinforce the argument that cnidarian photoreceptors are either precursors to or sharing a common ancestor with vertebrate ones. In contrast, the archetypical insect photoreceptor is of the rhabdomeric type. Cnidarian photoreceptors also share electrical methods of action with vertebrate photoreceptors; both types hyperpolarize when excited (Figure 2.)

The central cilium of these photoreceptors has projecting microvilli, which actually contain the stacked rhodopsin molecules (Kozmik 2003). The internal structure of the photosensory cell consists of a light-receptive outer region, a pigmented layer below it, and a nuclear layer furthest from the light input. Each pigmented cell has an associated neuron, which interconnects to those that surround it

to create an integrated perception of the surroundings. Cnidarian nerves are multifunctional structures In the medusoid species, the only specialized sensory structures are the cells of the ocelli and the statocysts. In the polyps, without such specialized structures, it may be hypothesized that the neurons take on these roles as well.

Those cnidarian species demonstrating photosensitivity manifest it most strongly in a marked phototacticity. Most freeswimming species will avoid dark objects and move toward shafts of light in the water. It has been speculated that this is both a feeding and a predator avoidance response. Many of these species live in near-shore environments, many of which are liberally filled with both obstacles and predators, both of which would appear to even simple light-dark photoreceptors as large dark spots. By contrast, many possible prey species might be found in the light patches, resulting in a light-following behavioral pattern likely to increase chances of both successful feeding and discovery of conspecifics. Some medusae have been shown to have a far more complex photoresponse than simply light-dark, as well. The irukandji, a small box jelly, displays a differential response to colored light, a response currently being investigated as a way to deter them from human-populated beaches.

Despite the nonspecific nature of the photoreceptors of the various polyp species, they display similar behaviors to the medusae. The photoresponse of hydra has been very well described, with hydra showing a marked movement into lighted areas and even a preference for some colors over others. The precise nature of the processing and cell types involved in this response is not yet fully understood.

Now that the structure of the discrete photoreceptors in some organisms and the behavior associated with the photoresponse are fairly well understood, attention has turned to the genomic pathways underlying the evolution, development, and maintenance of the responses. The early-evolving nature of the cnidarian photoreception systems makes them a prime target for understanding how photoreception as a whole came to be. Cnidarians are also significant because, in addition to their diverse photoreceptive structures, they display a broad variety of genes associated with vision, including all three types of opsins, C-, R-, and RGR/G_0 types (Lamb 2009). As hydra lack the pigmented cells thought typical of the photoresponse, of particular interest here is the role and presence of those genes tagged in other taxa as integral to the photoresponse, the Pax family.

The Pax gene family is a group of transcription factors named for the paired box domain they contain. The paired box domain is a region of 128 amino acids named for the Drosophila segment polarity gene in which it was first characterized, and featuring a distinctive serine residue at position 50 (Bopp 1986). The paired box domain has DNA binding activity in both the N- and C- terminal regions (Xu 1999). Many have a complete or partial homeodomain as well, and some also feature an octapeptide.

There are nine Pax genes in total in mammals and other more evolved taxa, grouped into four subfamilies (Callaerts 1997, Gehring 1999). Group one consists of *Pax1* and *Pax9* and is distinguished by a complete octapeptide. Group two, *Pax2*, *Pax5*, and *Pax8*, has the octapeptide and a partial homeodomain. Group three is *Pax3* and *Pax7*, and has both the complete octapeptide and a complete homeodomain.

Group four, the group of most interest in visual research, is *Pax4* and *Pax6*. These genes have a complete homeodomain, but no octapeptide.

The Pax genes have been heavily studied because of the significant role they play in development. *Pax 6,* discussed here, plays a crucial role in the formation of the eye, the central nervous system, the neural tube, and the olfactory epithelium (Stierwald nd, Simpson 2002). It has been highlighted as the master control gene for eye development; it triggers a genetic cascade that results in the formation of eyes. It also plays a role in defining the borders between regions of the central nervous system during the determination of the anterior-posterior axis (Mastick 1997). The differential effects in diverse tissues occur via the use of changing splice patterns specific to each tissue type, allowing for changes in the specific DNA binding activity and therefore affecting the precise control of expression of a phalanx of genes and proteins, including Six, Ets, Lim, Hes, Wnt, Maf, HLH, cadherins, keratins, and crystallins (Simpson 2002, Purcell 2005).

Interestingly, *Pax 6* is conserved to such a high degree that murine *Pax 6* can produce ectopic eyes in *Drosophila* that even appear to be somewhat functional in electrophysiological studies (Gehring 1999). Of even more interest when considering the significance of *Pax 6* in cnidarian photoreception is the finding that *PaxA-Cr*, the *Pax6* homologue in *Cladonema*, is also able to elicit ectopic eyes in *Drosophila* (Sun 2001). The eyes so formed remain *Drosophila*-specific, suggesting that the expression of a *Pax6* analog does not itself create the eye, but does suffice to turn on a developmental cascade that is specific to the genes present in the host organism. Some downstream targets of this cascade include the opsins, which have also been found in

hydra (Musio 2001, Plachetzki 2007).

Cnidarians appear to have between two and four Pax genes, usually referred to as *PaxA* through *PaxD* (Sun 1997, Bromham 2002, Plaza 2003). *Cladonema radiata* has three, A through C, while *Nematostella vectensis*, the starlet sea anemone, has four (Sun 2001, Matus 2007.) It has been speculated that hydra also may have as many as four. The high homology between *Nematostella* and *Hydra* sequences would seem to confirm this.

The homology between the PaxA-D genes of cnidarians and other nonmammalian taxa are not fully understood. It is thought that the nine mammalian genes arose from gene duplications after the split between coelenterates and the putative mammalian ancestor (Hoshiyama 1998). Sequence analysis of Pax genes collected from *Hydra littoralis* indicates that the hydra *PaxA* is closest in sequence to the *Pax4/6* group, with *H. littoralis' PaxB* mapping to the *Pax2/5/8* group. Conversely, however, analysis of the Pax genes found in *Hydra magnipapillata* hints at a more complex picture, with PaxB mapping to Pax4/6. A functional role has been proposed for *PaxB* that unites the roles of all three in vertebrates. This is similar to evolutionary sequence analysis as done by Shubin et al. (2009), which indicated that *PaxB* was, generally, the ancestral form of the visual *Pax 6* genes, with the later differentiation in structure and function in the vertebrates due to a duplication event after the cnidarian-vertebrate lineages split; see figure 1 for a tree. None of the hydroid genes have been fully functionally studied, however; the expression patterns and true developmental roles of each remain unclear. In this thesis, I will describe the discovery, sequencing, and localization of the *Hydra vulgaris PaxB* gene, with some

thoughts on its possible role in photoreception and neural development.

Photoreception requires more than simply the presence of a gene known to produce photoreceptive structures, however. The eye means nothing without the brain to interpret its signals, and hydra lack what most would categorize as a brain. They are tubular cnidarians with two epithelial layers, the endoderm and ectoderm. A ring of five to eight tentacles surround an oral pore, and the animal attaches to the substrate via adhesion of the basal disc at the opposite end. Hydra possess a complete ectodermal nerve net; non-nervous epithelial fingers interdigitate between the two layers, coordinating the functions of the ectodermal epithelial layer with those of the endoderm (Hufnagel and Kass-Simon 1976; Wood 1979). A proximal nerve ring surrounds the hypostome at the level of tentacle insertion and is considered to coordinate movements of the body, tentacles, mouth, and nematocysts in response to chemical, photic, and tactile stimuli (Kass-Simon 1972; Kinnamon 1981, Koizumi 1992). A second, possibly less-complete distal nerve ring is found inside that, more closely placed to the mouth (Hufnagel and Kass-Simon, unpublished).

Hydra have two contractile layers working in apposition: an internal layer of longitudinal myonemes, and an outer layer of circumferential myonemes (Scappaticci and Kass-Simon 2004). Contraction of the longitudinal myonemes contracts the body column; contraction of the circumferential myonemes results in an elongation. Illumination of the animal has been shown to result in their most unique behavior, the somersaulting response to strong stimulus. The animal elongates upward before reaching to one side. The isorhizas, a ‘sticky’ type of nematocyst, fire and attach the tentacles to the substrate; the opposing side of the body column then contracts,

flipping the basal disc over in the direction of travel. After reattachment of the basal disc, the animal then releases the isorhizal attachments and contracts the leading edge of the body to right itself. This process can continue until the animal has reached a more optimal environment.

There are three principal types of electrical activity seen in the hydra: pulses arising in the hypostome, pulses arising in the body, and pulses arising in the head (Passano and McCullough 1964). Any of these may be through-conducted to other body regions. The largest type of pulse is the tentacle or body pulse (TP and CP, respectively). These pulses, named for the site at which they are recorded, are large – even greater than 1mV – and are usually associated with the visible contraction of the associated body region. It is thought that both of these types of pulses originate in the hypostome (Passano and McCullough, 1964). Both TPs and CPs typically group together into bursts, the frequency of which has been found to vary under both extrinsic and intrinsic stimulation. Stimuli that change the rate of these bursts include light, time of day, degree of starvation, mechanical stimulation, electrical shock, and many neuromodulatory substances, including GABA, glutamate, glycine, NMDA, AMPA, atropine and others (Kass-Simon 1978, 2007).

Hydra have been known to be photosensitive since the 1800's, and have been shown to respond preferentially to some colors over others, with blue being preferred (Wilson 1891). They are sensitive to light applied in a narrow band on the base of the animal (Passano and McCullough 1964), as well as large-scale changes in the overall lighting of the environment. Exposure to light causes contractions of the endodermal musculature, resulting in body column extension (Passano and McCullough 1964).

Exposure to pulses of colored light also changes the frequency of both ectodermal contraction pulses and endodermal rhythmic waves in whole animal and head and tentacle preparations (Taddei-Ferretti et al. 2004). Previously reported experimental results have indicated that removal of the tentacles did not affect the frequency of light-elicited body column contractions, whereas removal of both the head and the tentacles resulted in a complete inhibition of such contractions (Rushforth 1963, 1971, 1973). Single excised tentacles are also independently photosensitive to strong white light, demonstrating that at least some part of the necessary photic inputs arise in the tentacles and are transmitted down the tentacles to the proximal hypostomal nerve ring (Passano and McCullough 1964). New to this study is the finding that the tentacles can also distinguish between different wavelengths of light.

Within the tentacles, the functional unit is the battery cell complex as described by Hufnagel and Kass-Simon (1985); see figure 3. The battery cell complex is a group of diverse cell types that together form an effector unit capable of responding to stimuli and transmitting that stimulus down the tentacle to the hypostomal nerve rings. It consists of large ectodermal epithelial cell, which surrounds and supports neurons and nematocytes. The nematocytes, in addition to responding to chemical, photic, and tactile stimuli while contained within the battery cell unit, have been shown to respond to photic and tactile stimuli when removed from it, showing that they possess simple sensor-effector capabilities (Kass-Simon and Scappaticci 2002, Scappaticci and Kass-Simon 2008, 2010.)

Differential photosensitivity between the tentacles and the whole animal implies a degree of as-yet-unquantified neuronal processing of the visual inputs. Because the

hydra nervous system is morphologically a simple network, describing the mechanisms by which such networks integrate sensory and effector signals may be profoundly important to determining how early brains evolved such integration.

References

Arendt, D. 2003. Evolution of eyes and photoreceptor cell types. *Int. J. Dev. Biol, 47*, 563-571.

Bentlage, B., Cartwright, P., Yanagihara, A., Lewis, C., Richards, G., Collins, A. 2009. Evolution of box jellyfish (Cnidaria: Cubozoa), a group of highly toxic invertebrates. *Proc Royal Society B.* 1-10.

Bopp D., Burri M., Baumgartner S., Frigerio G., Noll M. 1986. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. *Cell.* 47, 1033–1040.

Bromham, L. (2002). Searching for Pax in hydromedusa. *Trends in Ecology & Evolution, 17* (1), 11-12.

Callaerts, P., Halder, G., Gehring, W. 1997. Pax6 in development and evolution. *Ann. Rev. Neurosci*. 20, 483–532.

Coates, M. 2003. Visual Ecology and Functional Morphology of Cubozoa (Cnidaria). *Int Comp Bio.* 43 (4), 542.

Finnerty, J. 2001. Cnidarians reveal intermediate stages in the evolution of Hox clusters and axial complexity. *Amer Zool.* 41, 608-620.

Gehring, W., Ikeo, K. 1999. Pax 6: mastering eye morphogenesis and eye evolution. *Trends in Genetics,* 15 (9), 371-7.

Hoshiyama, D., Suga, H., Iwabe, N., Koyanagi, M., Nikoh, N., Kuma, K., Matsoda, F., Hongo, T., Miyata, T. 1998. Sponge Pax cDNA related to Pax 2/5/8 and ancient

gene duplications in the Pax family. *J. Mol. Evol*. 47: 640-648.

Hufnagel, L., Kass-Simon, G. The ultrastructural basis for the electrical coordination between epithelia of hydra. *Coelenterate Ecology and Behavior: Selected Papers* (1976).

Kass-Simon, G. 1970. Multiple excitation sites and straight-line conduction in the contraction burst system of Hydra. *Am. Zool.,* 10: 505. (Abstract).

Kass-Simon, G. 1972. Longitudinal conduction and contraction burst pulses from hypostomal excitation loci in Hydra attenuata. *J. Comp. Physiol*. 80: 29–49.

Kass-Simon, G. 1973. Transmitting systems in Hydra. *Publ. Seto Mar. Biol. Lab*. 20: 583–593.

Kass-Simon, G., Passano, L. 1978. A neuropharmacological analysis of the pacemakers and conducting tissues of Hydra attenuata. *J. Comp. Physiol. Part A.,* 128, 71-79.

Kass-Simon, G., Pierobon, P. 2007. Cnidarian neurochemical transmission: an updated overview. *Comp. Biochem. Physiol. Part A.,* 146: 9 –25.

Kass-Simon, G., Scappaticci, A. A. 2002. The behavioral and developmental physiology of nematocysts. *Can. J. Zool.,* 80: 1772– 1794.

Kinnamon, J. C., Westfall, J. A. 1981. A three dimensional serial reconstruction of neuronal distribution in the hypostome of Hydra. *J. Morphol*., 168: 321–329.

Koizumi, O., M. Itazawa, H. Mizumoto, S. Minobe, L. Javois, C. J. P.

Grimmelikhuijzen, Bode, H. R. 1992. Nerve ring of the hypostome in Hydra. I. Its structure and maintenance. *J. Comp. Neurol.,* 326: 7–21.

Kozmik, Z., Daube, M., Frei, E., Norman, B., Kos, L., Dishaw, L. 2003. Role of Pax genes in eye evolution: a cnidarian PaxB gene uniting Pax2 and Pax6 functions. *Dev Cell., 5* (5), 773-85.

Kozmik, Z., Ruzickova, J., Jonasova, K. 2008. Assembly of the cnidarian camera-type eye from vertebrate-like components. *PNAS.,* 105: 26; 8989-8993.

Lamb, T.D. 2009. Evolution of vertebrate retinal photoreception. *Philos Trans R Soc Lond B Biol Sci*. 364. 2911-24.

Martin, V. (2002). Photoreceptors of cnidarians. *Can J Zoo. 80* (10), 1703-1722.

Mastick G.S., Davis N.M., Andrew G.L., Easter S.S. Jr. 1997. Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. *Development*. 124: 1985 – 1997.

Matus, D., Pang, K., Daly, M., Martindale, M. 2007. Expression of Pax gene family members in the anthozoan cnidarian, *Nematostella vectensis*. *Evolution & development, 9* (1), 25-38.

Musio, C., Santillo, S., Taddei-Ferretti, C., Robles, L., Vismara, R., Barsanti, L., Gualtieri, P. 2001. First identification and localization of a visual pigment in Hydra (Cnidaria, Hydrozoa). *J Comp Phys A.,* 187 (1), 79-81.

Passano, L. M., McCullough, C.B. 1962. Light response and the rhythmic potentials

of *Hydra*. *PNAS*., 48: 1376– 1382.

Passano, L. M., McCullough, C.B. 1963. Pacemaker hierarchies controlling behavior of *Hydra*. *Nature,* 199: 1174–1175.

Passano, L. M., McCullough, C.B. 1964. Coordinating systems and behavior in *Hydra*. I. Pacemaker system of the periodic contractions. *J*. *Exp*. *Biol*. 41: 643–644.

Passano, L. M., McCullough, C.B. 1965. Coordinating systems in *Hydra:* the rhythmic potential system. *J*. *Exp*. *Biol*. 42: 205–231.

Plachetzki, D., Degnan, B., Oakley, T. 2007. The Origins of Novel Protein Interactions during Animal Opsin Evolution. *PLoS ONE*, 10: e1054.

Plaza, S., De Jong, D., Gehring, W., Miller, D. 2003. DNA-binding characteristics of cnidarian PaxC and PaxB proteins in vivo and in vitro: no simple relationship with the Pax6 and Pax2/5/8 classes. *J. Exp. Zoo. B.,* 299 (1), 26-35.

Purcell, P., Oliver, G., Mardon, G., Donner, A., Maas, R. 2005. Pax6-dependence of Six3, Eya1 and Dach1 expression during lens and nasal placode induction. *Gene Expression Patterns*, 6: 110–118.

Rushforth, N. B., Burke, D. S. 1971. Behavioral and electrophysiological studies of *Hydra*. II. Pacemaker activity of isolated tentacles. *Bio Bull*., 140: 502–519.

Rushforth N, Burnett A, Maynard R. 1963 Behavior in hydra: Contraction responses of Hydra pirardi to mechanical and light stimuli. *Science*., 139: 760-761.

Rushforth N, Hofman F. 1972. Behavioral and electrophysiological studies of hydra.

III. Components of feeding behavior. *Bio Bull*., 142:110-131.

Scappaticci, A.A., Kass-Simon, G. 2008**.** NMDA and GABAB receptors are involved in controlling nematocyst discharge in hydra. *Comp Biochem Physiol A.,* 150: 415–422.

Scappaticci A.A., Kahn F., Kass-Simon G. 2010. Nematocyst discharge in Hydra vulgaris: Differential responses of desmonemes and stenoteles to mechanical and chemical stimulation. *Comp Biochem Physiol, Part A*., 157:184–191.

Shubin, N. Tabin, C, Carroll, S. 2009. Deep homology and the origins of evolutionary novelty. *Nature*., 147:12, 818-823.

Singer, R., Rushforth, N., Burnett, A. 1963. The Photodynamic Action of Light on Hydra. *J Exp Zoo,* 154, 169-73.

Stierwald, M. (n.d.). On The Evolution Of Cnidarian Eyes. *unibas.ch*; *Doctoral thesis*.

Sun, H., Dickinson, D., Costello, J., Li, W. 2001. Isolation of Cladonema PaxB genes and studies of the DNA-binding properties of cnidarian Pax paired domains. *Molecular Biology and Evolution., 18* (10), 1905-18.

Sun, H., Rodin, A., Zhou, Y., Dickinson, D., Harper, D., Hewett-Emmett, D. 1997. Evolution of paired domains: isolation and sequencing of jellyfish and hydra Pax genes related to Pax5 and Pax6. *PNAS,. 94* (10), 5156-61.

Xu, H.E., Rould, M.A., Xu, W., Epstein, J.A., Maas, R.L., Pabo, C.O., 1999. Crystal structure of the human Pax6 paired domain-DNA complex reveals specific roles for

the linker region and carboxy-terminal subdomain in DNA binding. *Genes Dev.,* 13, 1263-1275.

Fig. 1

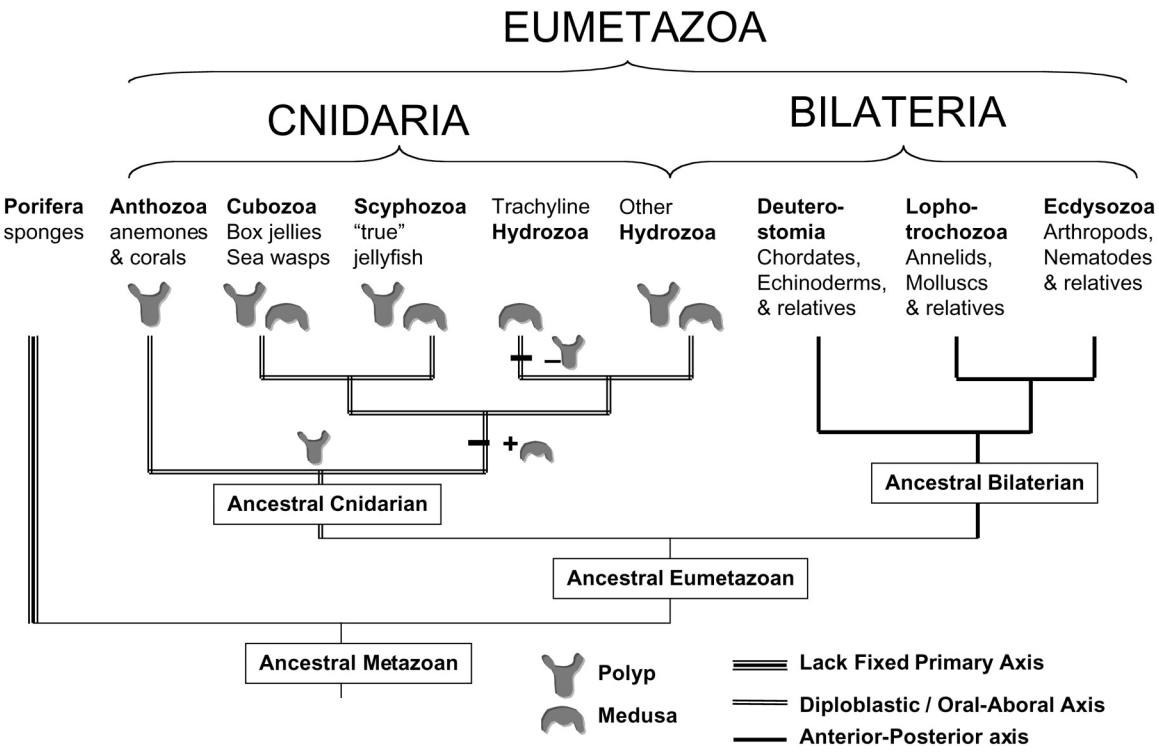

EUMETAZOA
CNIDARIA
BILATERIA
Porifera sponges
Anthozoa anemones & corals
Cubozoa Box jellies Sea wasps
Scyphozoa "true" jellyfish
Trachyline Hydrozoa
Other Hydrozoa
Deutero-stomia Chordates, Echinoderms, & relatives
Lopho-trochozoa Annelids, Molluscs & relatives
Ecdysozoa Arthropods, Nematodes & relatives
Ancestral Cnidarian
Ancestral Bilaterian
Ancestral Eumetazoan
Ancestral Metazoan
Polyp
Medusa
Lack Fixed Primary Axis
Diploblastic / Oral-Aboral Axis
Anterior-Posterior axis

Fig. 2

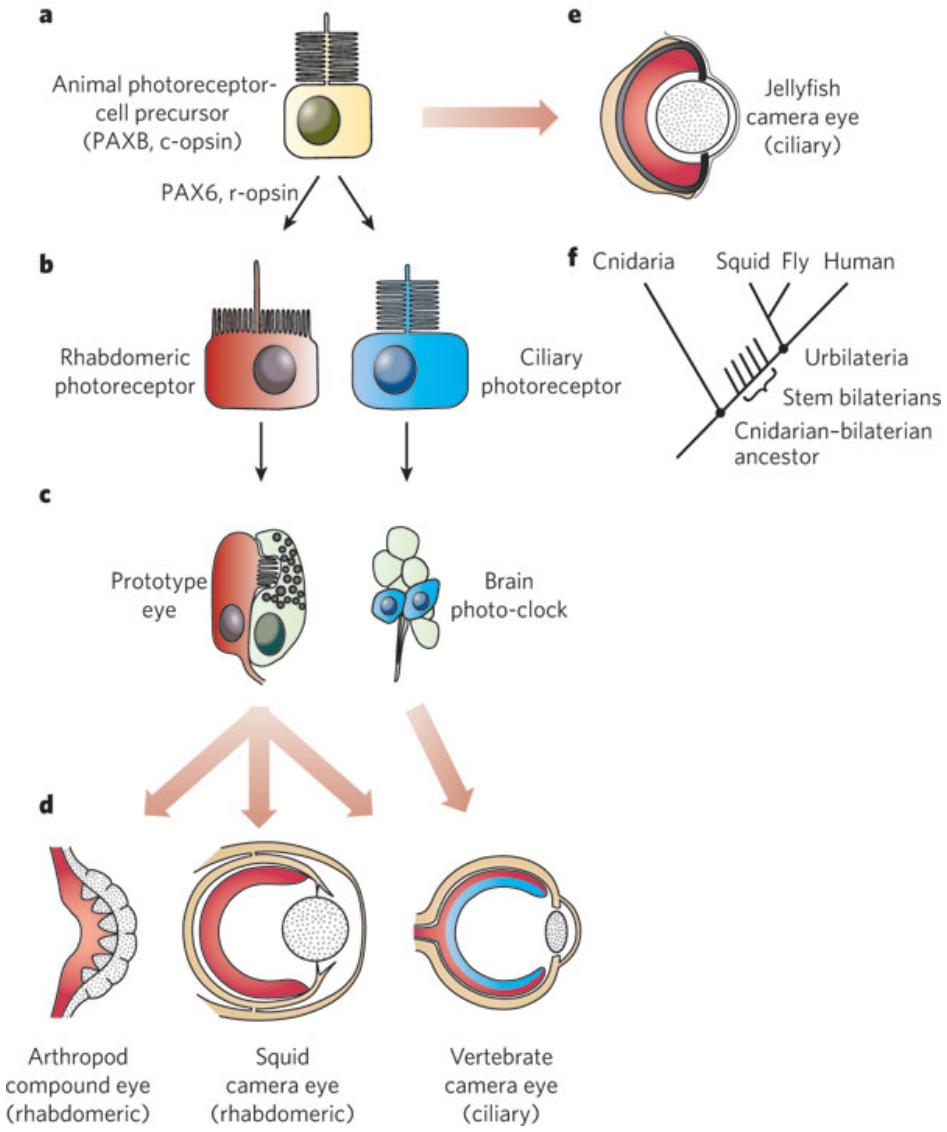
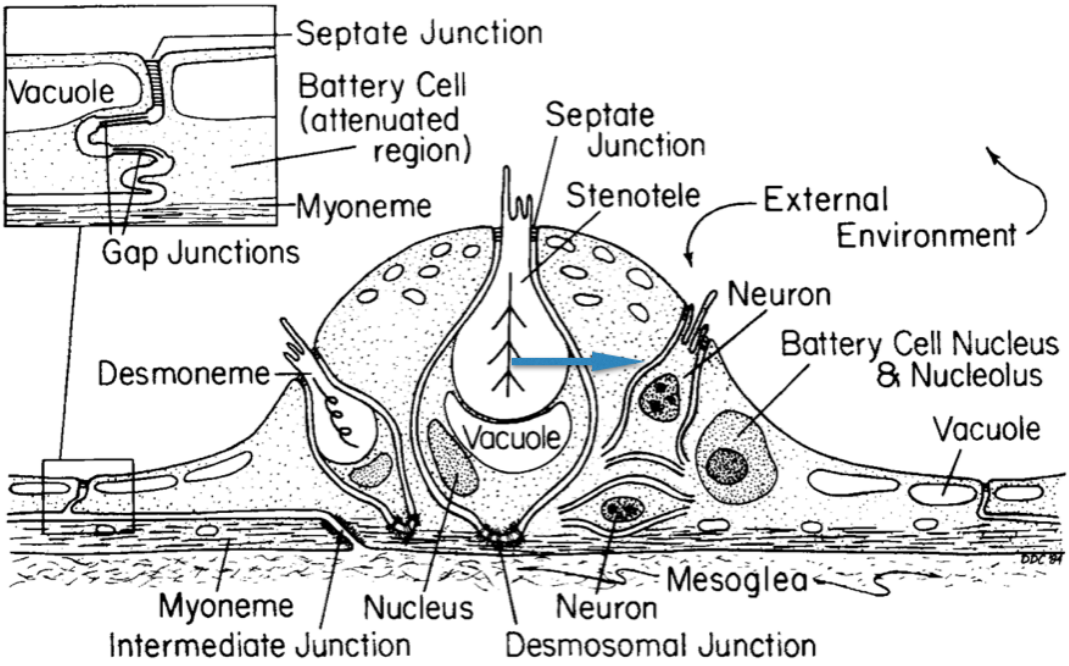

a
Animal photoreceptor-cell precursor (PAXB, c-opsin)
PAX6, r-opsin
e
Jellyfish camera eye (ciliary)
b
Rhabdomeric photoreceptor
Ciliary photoreceptor
f
Cnidaria
Squid
Fly
Human
Urbilateria
Stem bilaterians
Cnidarian-bilaterian ancestor
c
Prototype eye
Brain photo-clock
d
Arthropod compound eye (rhabdomeric)
Squid camera eye (rhabdomeric)
Vertebrate camera eye (ciliary)

Fig. 3


Septate Junction
Vacuole
Battery Cell
(attenuated
region)
Septate
Junction
Stenotele
External
Environment
Myoneme
Gap Junctions
Neuron
Battery Cell Nucleus
& Nucleolus
Desmoneme
Vacuole
Vacuole
Mesoglea
Myoneme
Intermediate Junction
Nucleus
Neuron
Desmosomal Junction

CHAPTER 2

HvPaxB Expression in Regenerating and Budding Hydra vulgaris

Stephanie L. Guertin[2], Gabriele Kass-Simon[1,2], and Steven Q. Irvine[1*]

[1]Department of Biological Sciences, College of the Environmental and Life Sciences, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881-0816

[2]Interdisciplinary Neurosciences Program, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881-0816

* Author for correspondence (email: sirvine@uri.edu)

In preparation for submission to:

Development, Genes, and Evolution

Abstract

The early-evolved metazoan hydra has a complex sensory system that responds to chemical, mechanical, and photosensory stimuli. Despite hydra's complex responses to light, little is known about the development of the cellular basis for its light perception. Here we report the cloning and localization of the *Pax B* gene in *Hydra vulgaris*. The sequence was isolated from cDNA via PCR and confirmed by phylogenetic analysis. Using whole mount in situ hybridization, specific labeling of presumptive nematocytes and interstitial cells in whole animals, 24-hour regenerates, and mid- to late-stage buds was found. Labeling was concentrated at the apical end in regenerates and buds, and found in cells scattered throughout the adult body column. Together with earlier behavioral findings, the localization of HvPaxB transcripts in budding and regenerating animals suggests that this gene plays a role in neural organization and the specification of phototransduction structures in hydra. The time-dependent expression in structures destined to be crucial to light perception suggests that these structures are specified during a finite phase in morphogenesis.

Background

Hydrozoans are considered the best extant representative of the ancestral metazoan form; they have a simple tubular body plan, unsegmented appendages, and a simple nervous system. The genome of the anthozoan *Nematostella vectensis* is thought to have diverged relatively little from its metazoan common ancestor, implying that hydra, as its near relative, may also have characteristics of the most primitive animals (Putnam et al 2007).

Hydras consist of two epithelial layers, the endoderm and ectoderm, separated by an acellular mesoglea. A ring of five to eight tentacles surrounds a hypostome, at the apex of which, is an oral pore. Hydra possess a complete ectodermal nerve net, although its endodermal net is said to consist only of loosely connected fibers (Westfall 1971). Non-innervated epithelial projections interdigitate between the two layers, providing a pathway for coordination between the layers (Hufnagel and Kass-Simon 1976, Wood 1979). A nerve ring of interconnected fibers surrounds the mouth in *Hydra magnipapillata* and other species of hydra that has been suggested to coordinate the feeding behavior in the hypostome (Westfall 1971, Koizumi 2007). A second nerve ring, running between and below the tentacles, coordinates tentacular and body contraction behavior in response to chemical, photic, and tactile stimuli (Kass-Simon 1972).

Hydra have been known to be photosensitive since the 1800's, although they have neither eyes nor other conventional photoreceptive structures. The *Hydra pirardi* used in Wilson's experiments congregated preferentially in regions of a tank lit with blue light rather than those lit with other colors. Several species of hydra (*H. pirardi,*

H. oligactis, & H. littoralis) are sensitive to light applied in a narrow beam to the base of the animal (Passano and McCullough 1962). Exposure to light causes contractions of the endodermal musculature and changes the frequency of ectodermal contraction and endodermal electrical impulses (Passano and McCullough 1964, Singer et al 1963, Taddei-Ferretti 2004). Taddei-Ferretti also describes a bioelectric "big slow wave" that correlates to the contraction-extension behavior of the body column and which is affected in *H. vulgaris* by stimuli of 400 to 500 nanometer light, with sensitivity decreasing above 500 nm but returning slightly at wavelengths above 575 nm.

Plachetzki et al (2012) have recently shown that light is involved in the regulation of nematocyst firing through the activation of cyclic-nucleotide gated channels (CNG). When cells expressing opsin along with CNG genes were exposed to light, the associated nematocysts fired. Knockout experiments on the CNG channels showed that these and their associated light response were necessary for the firing.

It is therefore desirable to examine other links in a putative photosensory cascade. One such link involves the Pax family of transcription factors, which have been implicated in direct photosensory specification and in the nervous pathways that process light stimuli. In mammals, there are nine Pax genes, numbered 1 through 9. In cnidarians, the number of Pax genes remains unclear, with numbers as high as nine in some species (Matus et al 2007).

Relationships between the Pax gene network in complex organisms and those in more basal organisms have been sought. A pair of Pax genes, PaxA and PaxB, were found in sea nettles and hydra (Sun et al 2001). The starlet sea anemone, *Nematostella*

vectensis, has nine Pax genes, while the coral *Acropora millepora* has four (Matus et al 2007). In addition, the protein products of hydra PaxA bind to a site for Pax-5/6 products, suggesting that the protein was highly conserved as the various taxa evolved. Structural analysis and binding affinity experiments on *Hydra*, *Cladonema*, and *Tripedalia* indicate that cnidarian PaxB is the orthologue of the Pax 2/5/8 subfamily in bilaterians (Sun et al 2001, Kozmik et al 2003, Suga et al 2010). In the hydroid jellyfish *Podocoryne carnea*, PaxB plays a role in the differentiation of nerve cells (Gröger et al 2000). The PaxB gene from the cubozoan *Tripedalia cystophora*, has characteristics of both the Pax2/5/8 and Pax6 subfamilies (Kozmik et al 2003). Pax6, which may be similar to cnidarian PaxA, is a photosensitivity-related gene implicated in the early stages of the formation of photoreceptive cells and structures and is found across taxa from cnidarians to humans.

This highly conserved Pax gene codes for a transcription factor instrumental in the formation of eyes and other tissues. It has been proposed that it has been necessary for the formation of photoreceptor cells since the earliest metazoan ancestors (Arendt 2003). In fruit flies, ectopic expression of the Pax6 protein product causes the production of eyes on the legs or antennae (Halder et al 1995, Callaerts et al 1997). The PaxA gene of *Cladonema californicum*, a scyphozoan jellyfish, is capable of inducing ectopic eyes in Drosophila (Suga et al 2010). Remarkably, PaxB from another scyphozoan, *Tripedalia cystophora,* can also induce ectopic eyes in flies (Gröger et al 2000).

Here we present evidence that the PaxB gene found in *H. vulgaris* appears during development of body regions and cell types involved in the animal's sensory

system.

Methods

Animal culture

Specimens of *Hydra vulgaris* were asexually cultured in glass baking dishes containing modified bicarbonate versene culture solution (BVC): 1 x 10 - 7 mol/L NaHCO3, 1 x 10-6 mol/L $CaCl_2$, and 1 x 10-8 mol/L EDTA, pH 7.0 +/- 0.2 (Loomis and Lenhoff 1956, Muscatine and Lenhoff 1965). All animals were kept in an incubator at 18 +/- 1.0 °C. Hydra cultures were fed freshly hatched *Artemia salina* nauplii once every 48 h.

Experimental preparation

To prevent possible effects of feeding on gene expression, experimental animals were randomly selected from non-budding hydra that had been starved for 24 +/- 2 h before being selected for subsequent preparation. All animals were relaxed in 2% menthol in hydra medium and fixed overnight at 4°C in 4% paraformaldehyde in hydra medium (Hufnagel et al 1985).

For regenerating animals, starved animals were cut and allowed to regenerate for 0, 12, 24, or 48 hours.

For budding animals, starved animals bearing buds of various stages were fixed; the age of the bud was determined by size and level of development as follows:

Stage 1: earliest appearance of identifiable protobud, the anlage

Stage 2: lateral extension of tissue that is discrete from parent

Stage 3: first emergence of tentacle stubs

Stage 4: bud is nearly adult, tentacle stubs extended, but not fully grown, bud

may have begun to bend downward prior to separation.

See Fig 1 for bud stages.

Cloning *HvPaxB*

To isolate the gene, mRNA was extracted from an aggregate of 200 whole, nonbudding animals. PCR was run on this cDNA, using primer sequences that were made from *H. magnipapillata* published sequences and custom ordered from Invitrogen. The primers used were HvPaxBF1, 5'-TGAGTTGGCACATCAAGGTGTCCG and HvPaxBR1, 5'-CAAAAGCATCTTCAAGGGCTCTGC..

Phylogenetic analysis

Clustal Omega with default settings was used to align the HvPaxB sequence to 20 other Pax gene sequences (Sievers et al 2011). Regions with quality scores below 6 were manually removed from the alignment to produce 117 concatenated amino acid positions, including the paired domain. Alignment is included as Supplemental Figure 1. Neighbor-joining and maximum likelihood trees were constructed using the JTT model of evolution and 1000 bootstrap replicates using the Phylip 3.695 Neighbor and ProtML programs after making datasets with ProtDist and SeqBoot: see figure 2 for Neighbor and supplemental figure 2 for ProtML (Felsenstein 1989).

In situ hybridization

Whole-mount in situ hybridization was performed with digoxigenin-labeled sense and antisense probes using the protocols of Bridge and Galliot (Bridge et al 2000, Galliot 2012). Probes were heated for 5 min to remove secondary structure prior to their addition to the hybridization solution. Hybridization was performed at 55°C. The probe was made against *HmPaxB*, a sequence obtained from KEGG [KEGG:100192231**]** and confirmed by PCR in our animals (Kanehisa and Goto, 2000).

For light microscopic sections: Animals were refixed and embedded in Spurr's resin, then sectioned into 1 μm sections using a glass knife. These sections were fixed on slides and examined under oil immersion (100x) with an Olympus BX51 microscope.

Results

Cloning an *H. vulgaris* Pax gene and relationship to other Pax genes

Our Clustal Omega alignments were used to generate phylogenetic trees using the ProtML (Fig.1) and Neighbor (Supplemental Fig. 1) programs in the Phylip 3.695 package (Felsenstein 1989). In both methods *HvPaxB* grouped with the Pax2/5/8 genes, along with other cnidarian PaxB genes, indicating that our initial orthology assessment was correct.

Transcript expression in whole animals

We used whole mount *in-situ* hybridization (ISH) to determine the mRNA expression pattern of *HvPaxB*. Of 11 fixed animals, 11 showed positive signal from the antisense digoxygenin-labeled *in-situ* hybridization probe (table 1). In adult, non-budding, non-regenerating hydra, expression was seen in scattered cells whose morphology suggests that they are neuronal precursors and clusters of developing nematoblasts in the body column (Fig. 3, 4). These groups of interstitial cells, similar to those identified by David and Bridge, consist of "nests" of small, oblate to circular cells that tend to be visible in multiples of two (David and Challoner 1974, Bridge et al 2010). Later in development, these cells exhibit processes emanating from a central cell body, in keeping with the known morphology of neuronal precursors (David and Challoner 1974). Staining was also observed in cell groups that appear to be developing battery cell complexes (Fig. 9), with the nascent nematocyst clearly unstained within its supporting cell. No broad ISH signal was detected in the hypostome, tentacles, or foot.

Transcript expression in regenerating animals

Regenerating animals showed strong *HvPaxB* ISH signal at the site of head regrowth (Fig 5, 6 for detail). When fixed immediately after decapitation, no expression was seen in any animals. The strongest and most consistent expression (6/8 animals) appeared at 24 hours after decapitation. The first evidence of expression appeared at 12 hours after decapitation in the regenerating head *anlage*, which at this time is approximately the upper fifth of the body column. This expression was less frequent and less intense than that at 24 hours, with expression appearing in only 2/10 animals - presumably due to lower levels of transcript within the cells. Expression was also more diffuse in the early stages of regeneration; expression at 24 hours was seen as a narrower, stronger band across the apical end of the animal. By 48 hours, transcript expression was no longer evident.

Transcript expression in budding animals

The results in the regenerating and adult animals indicated that *HvPaxB* might be most strongly expressed during periods of growth and reorganization. We assayed the expression in budding animals at various developmental stages (Table 1). No ISH signal was detected in Stage1 buds (0 of 8 animals.) In Stage 2, only 1/9 animals had detectable expression. The strongest expression was found in the Stage 3 and 4 buds. In Stage 3, 9/12 of fixed animals showed expression. In particular, the tentacle buds in Stage 3 had strong *HvPaxB* ISH signal as they began to protrude from the main body of the bud (Fig 7).

Transcript expression in thin sections

To verify tissue localization in budding and regenerating animals, we imaged 1 μm thin sections, cut from whole-mount ISH specimens (Fig 8), which, confirmed cytoplasmic staining, even in isolated cells. It was possible to identify several cell types. Staining was visible in putative battery cell complexes (Fig 9), as well as several types of neuronal cells. Staining was also present in the buds of Stage 4 budding animals in the region immediately surrounding the foot of the bud, and in the associated adult, immediately proximal to the bud.

Discussion

Our findings indicate that the strongest expression of *HvPaxB* is concurrent with the early formation of tentacles and during the organization of the tentacular battery cell complexes (Bode and David 1978). In the developing buds, although nascent tentacles expressed *HvPaxB*, not all did so at the same time. Our data suggest that *HvPaxB* is expressed at high levels only for a short time during tentacle development. Expression in mature animals was very low, consistent with Pax's putative role mainly in development. Hydra have pluripotent stem cells called interstitial or i-cells which are localized primarily in the region below the head, but are found throughout the body column and identified by shape and size and have the ability to diversify into several lineages of cell types (David and Challoner 1974, Bode 1996.). One cell type, identified by David as the nematoblast, develops into nematocytes and neural precursors (David and Challoner 1974, Bridge et al 2010, Galliot et al 2009). In many of our specimens, these late-stage neuronal-lineage i-cells - identified as small oblate cells occurring in clusters - exhibited *HvPaxB* transcript expression (Fig 4).

Expression was seen in i-cells and sensory cells in adults, and in regions inhabited by these cell types in regenerating animals. Expression was not seen in mature nematocysts, but rather in the surrounding nematocytes (Fig 6 and 9). The timing and location of *HvPaxB* expression in our experiments is consistent with the idea that *HvPaxB* may be involved during the organization of the bud and tissue reorganization of the regenerating head. *HvPaxB* staining was consistent in timing and location with the activity of other genes implicated in axial patterning in hydra, such

as *HyWnt* and *HyTcf* (Hobmayer et al 2000, Broun and Bode 2002).

Particularly of note is the expression of PaxB in battery cells surrounding the non-staining mature nematocysts (Fig 6 and 9). Battery cells have been implicated in chemo- and mechanosensory reception and light perception (Kass-Simon and Scappaticci 2004, Scappaticci et al 2010, Plachetzki et al 2012). The coexpression of CNG and opsin genes in these sensory cells seen in recent work further indicates a role for them in photoreception (Plachetzki 2012). Also found to express PaxB were a variety of putative neuronal cells, of several different types, both closely associated with battery cell complexes and more widely scattered throughout the body. In bilaterians, Pax genes have roles in the organization of the nervous system, particularly in the specification of the anterior-posterior axis (Gehring and Ikeo 1999). The nervous system of hydra appears not to have the same single linear axis as in bilaterians; however, evidence indicates that there is an apical-basal differentiation in its function (Rushforth and Burke 1971, Westfall et al 1971, Kass-Simon 1972, Rushforth and Hofman 1972, Grimmelikhuijzen 1985, and Koizumi 200). It is possible that in hydra too, the *HvPaxB* gene is instrumental in defining the body regions that will react to a photic stimulus.

Expression was seen in clusters of small, oblate cells scattered throughout the body, cells visually identified as the developing nematocytes, or nematoblasts, described in previous research (Bode and David 1978, Bridge et al 2010). These cells have been shown to develop in epithelial niches in the ectoderm (Bosch and David 1990, Bosch 2008). The clusters have been identified specifically as those i-cells that form neuro- and nematoblast cell lineages. Nematocytes in hydra's tentacles have

been shown to respond to electromagnetic, chemical and, mechanical stimuli as well as to traditional neurotransmitters (Kass-Simon 1973, Kass-Simon and Scappaticci 2004, Thurm et al 2004, Scappaticci and Kass-Simon 2008, and Plachetzki et al 2012). Since nematocytes also develop from the neuroblast precursors (David and Challoner 1974, Bridge et al 2010), expression of *HvPaxB* in these cells is yet another indication that *HvPaxB* is involved in the specification of sensory and effector cells in hydra.

Scattered throughout the bodies of all our specimens were examples of putative neuronal precursors, identified by their shape, size, and extending processes. In general, two to three such cells were evident in the body column in all preparations, even non-regenerating, non-budding adults. We surmise that these cells are replacement cells or new cells contributing to a growing animal.

If it is true that *HvPaxB* is uniquely expressed in developing neural elements, then, with respect to budding animals, expression at the bud's foot shortly before detachment from the parent hints at a neural concentration in the foot in the adult. This correlates with the previous findings that light-induced behavioral changes can originate in the foot and that light directed to hydra's foot affects rhythmic potential impulses (Passano and McCullough 1962, Rushforth et al 1963, Singer et al 1963,Taddei-Ferretti and Musio 2000). Expression in closely associated cells of the parent animal at the time that the bud is detaching suggests that there may be some organizing influence on the formation of the foot of the bud from the parent, or that the parent is reorganizing its own sensory cells in preparation for the separation of the bud. The possible influence in foot organization is consistent with the idea of a foot

organizer in hydra (Hicklin and Wolpert 1973).

Conclusions

In summary, we have found that *HvPaxB* variously expressed in the asexual development and regeneration of the hydra. In the adult, expression is seen in scattered cells throughout the body, cells that may be interstitial cells of the developing neuronal type. It is also present at the site of head regrowth in 24-hour regenerating animals. In the developing bud, expression is found in the tentacle stubs of mid-stage buds, and in the foot region of late-stage buds.

The transcript expression pattern of *HvPaxB* in hydra indicates that, as with Pax genes in other organisms, this gene may play a significant role in the organization of the nervous and photosensitive systems.

Acknowledgements

We gratefully acknowledge Dr. Alison W. Roberts' assistance with the thin section preparation and microtome training. We also thank Joanna Ying, undergraduate student in the Department of Biology, for assistance in performing the in situs.

SG received a URI Graduate Research grant towards purchase of the reagents for the in situ protocol.

Literature Cited

1. Arendt D: **Evolution of eyes and photoreceptor cell types**. *Int J Dev Biol* 2003, **47**:563–571.
2. Bode H: **The interstitial cell lineage of hydra: a stem cell system that arose early in evolution.** *J Cell Sci* 1996, **109**:1155–1164.
3. Bode, H, David, CN: **Regulation of a multipotent stem cell, the interstitial cell of hydra**. Prog Biophys Mol Biol 1978, 33: 189-296.
4. Bosch T, David C: **Cloned interstitial stem cells grow as contiguous patches in hydra**. *Dev Biol* 1990:513–515.
5. Bosch T: **Stem cells in immortal hydra**. *Stem Cells: From Hydra to Man* 2008:37–57.
6. Bridge D, Theofiles AG, Holler RL, Marcinkevicius E, Steele RE, Martinez DE: **FoxO and stress responses in the cnidarian *Hydra vulgaris*.** *PLoS ONE* 2010, **5**: e11686.
7. Bridge DM, Stover NA, Steele RE: **Expression of a novel receptor tyrosine kinase gene and a paired-like homeobox gene provides evidence of differences in patterning at the oral and aboral ends of hydra.** *Dev Biol* 2000, **220**:253–262.
8. Broun M, Bode HR: **Characterization of the head organizer in hydra.** *Development* 2002, **129**:875–884.
9. Callaerts P, Halder G, Gehring W: **Pax-6 in development and evolution**. *Annual Reviews in Neuroscience* 1997:483–532.

10. David CN, Challoner D: **Distribution of interstitial cells and differentiating nematocytes in nests in *Hydra attenuata***. *Am Zool* 1974, **14**:537–542.

11. Felsenstein, J: **PHYLIP - Phylogeny Inference Package (Version 3.2).** *Cladistics* 1989, **5:** 164-166.

12. Galliot B, Quiquand M, Ghila L, de Rosa R, Miljkovic-Licina M, Chera S: **Origins of neurogenesis, a cnidarian view.** *Dev Biol* 2009, **332**:2–24.

13. Galliot B: **Injury-induced asymmetric cell death as a driving force for head regeneration in hydra**. *Roux's Arch Dev Biol* 2012, **223**:39–52.

14. Gehring W, Ikeo K: **Pax 6: mastering eye morphogenesis and eye evolution**. *Trends in Genetics* 1999, **15**:371–377.

15. Grimmelikhuijzen C: **Antisera to the sequence Arg-Phe-amide visualize neuronal centralization in hydroid polyps**. *Cell Tissue Res* 1985, **241**:171–182.

16. Gröger H, Callaerts P, Gehring WJ, Schmid V: **Characterization and expression analysis of an ancestor-type Pax gene in the hydrozoan jellyfish *Podocoryne carnea*.** *Mechanisms of Development* 2000, **94**:157–169.

17. Guertin, S, Irvine, SQ, Nandivada, V, Hufnagel, LA, Kass-Simon, G: **Extraocular photosensitivity in hydra tentacles: Electrophysiological manifestations and genomic evidence**. In Proceedings of the Annual Meeting of Society for Neuroscience: 17-22 October 2009.

18. Halder G, Callaerts P, Gehring WJ: **New perspectives on eye evolution.** *Curr Opin Genet Dev* 1995, **5**:602–609.

19. Hicklin J, Wolpert L: **Positional information and pattern regulation in hydra: formation of the foot end.** *J Embryol Exp Morphol* 1973, **30**:727–740.

20. Hobmayer B, Rentzsch F, Kuhn K, Happel CM, Laue von CC, Snyder P, Rothbächer U, Holstein TW: **WNT signalling molecules act in axis formation in the diploblastic metazoan hydra.** *Nature* 2000, **407**:186–189.

21. Hufnagel L, Acevedo, S, Soucy, B, Su K, Kass-Simon G: **Circumferential nerve rings in the hypostome of *Hydra vulgaris*.** In Proceedings of the International Workshop on Basal Metazoa: 12-15 September 2011; Edited by Thomas C. G. Bosch and Thomas W. Holstein: 2011: 127.

22. Hufnagel L, Kass-Simon G, Lyon MK: **Functional organization of battery cell complexes in tentacles**. *J Morphol* 1985, **184**:323-341.

23. Hufnagel L, Kass-Simon G: **The ultrastructural basis for the electrical coordination between epithelia of hydra**. In *Coelenterate Ecology and Behavior: Selected Papers.* Edited by Mackie GO. New York: Plenum Press; 1976: 695-705.

24. Kanehisa, M, Goto, S: **KEGG: Kyoto encyclopedia of genes and genomes.** Nucleic Acids Res 2000, **28**:27-30.

25. Kass-Simon G, Passano L: **A neuropharmacological analysis of the pacemakers and conducting tissues of *Hydra attenuata***. *J Comp Phys A: Sensory* 1978, **128**:71-79.

26. Kass-Simon G, Scappaticci A: **Glutamatergic and GABAnergic control in the tentacle effector systems of *Hydra vulgaris***. *Hydrobiologia* 2004, **530/531**: 67-71.

27. Kass-Simon G, Scappaticci AA: **The behavioral and developmental physiology of nematocysts**. *Can J of Zool* 2002, **80**:1772–1794.

28. Kass-Simon G: **Coordination of juxtaposed muscle layers as seen in hydra**. In *Coelenterate Ecology and Behavior: Selected Papers.* Edited by Mackie GO. New York: Plenum Press; 1976:705-713.

29. Kass-Simon G: **Longitudinal conduction of contraction burst pulses from hypostomal excitation loci in *Hydra attenuata***. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology* 1972, **80**:29–49.

30. Kass-Simon G: **Transmitting systems in hydra**. Publ Seto Mar Biol Lab 1973, **20:**583-594

31. Koizumi O: **Nerve ring of the hypostome in hydra: is it an origin of the central nervous system of bilaterian animals?** *Brain Behav Evol* 2007, **69**:151–159.

32. Kozmik Z, Daube M, Frei E, Norman B, Kos L, Dishaw LJ, Noll M, Piatigorsky J: **Role of Pax genes in eye evolution: a cnidarian PaxB gene uniting Pax2 and Pax6 functions.** *Dev Cell* 2003, **5**:773–785.

33. Loomis WF, and Lenhoff HM: **Growth and sexual differentiation of hydra in mass culture.** J Exp Zool 1956, 132: 555-573.

34. Matus D, Pang K, Daly M, Martindale M. **Expression of Pax gene family members in the anthozoan cnidarian, *Nematostella vectensis.*** Evol Dev 2007, **9**: 25-38

35. Muscatine L, Lenhoff H: **Symbiosis of hydra and algae. I. Effects of some environmental cations on growth of symbiotic and asymbiotic hydra.** *Bio Bull* 1965, **128:** 415-424.

36. Passano L, McCullough C: **Co-ordinating systems and behaviour in hydra: I. Pacemaker system of the periodic contractions**. *Journal of Experimental Biology* 1964, **41**:643-664.

37. Passano L, McCullough C: **The light response and the rhythmic potentials of hydra**. *Proc Natl Acad Sci USA* 1962, **48**:1376–1382.

38. Passano L, McCullough C: **The light response and the rhythmic potentials of hydra**. *Proc Natl Acad Sci* 1962:1376–1382.

39. Plachetzki DC, Fong CR, Oakley TH: **Cnidocyte discharge is regulated by light and opsin-mediated phototransduction**. *BMC Biol* 2012, **10**:17.

40. Putnam NH, Srivastava M, Hellsten U, Dirks B, Chapman J, Salamov A, Terry A, Shapiro H, Lindquist E, Kapitonov VV, Jurka J, Genikhovich G, Grigoriev IV, Lucas SM, Steele RE, Finnerty JR, Technau U, Martindale MQ, Rokhsar DS: **Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization**. *Science* 2007, **317**:86–94.

41. Rushforth N, Burke D: **Behavioral and electrophysiological studies of hydra. II. Pacemaker activity of isolated tentacles**. *The Biological Bulletin* 1971, **140**: 502-519.

42. Rushforth N, Burnett A, Maynard R: **Behavior in hydra: Contraction responses of *Hydra pirardi* to mechanical and light stimuli**. *Science* 1963, **139**: 760-761.

43. Rushforth N, Hofman F: **Behavioral and electrophysiological studies of hydra. III. Components of feeding behavior**. *The Biological Bulletin* 1972, **142**:110-131

44. Scappaticci A, Kass-Simon G: **NMDA and GABAB receptors are involved in controlling nematocyst discharge in hydra**. *Comp Biochem Phys* 2008, **150**: 415–422.

45. Scappaticci AA, Kahn F, Kass-Simon G: **Nematocyst discharge in *Hydra vulgaris*: Differential responses of desmonemes and stenoteles to mechanical and chemical stimulation.** *Comp Biochem Physiol, Part A Mol Integr Physiol* 2010, **157**:184–191.

46. Sievers F, Wilm A, Dineen DG, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG: **Fast, scalable generation of high quality protein multiple sequence alignments using Clustal Omega**. *Mol. Systems Biol.* 2011, **7**:539

47. Singer RH, Rushforth NB, Burnett AL: **The photodynamic action of light on Hydra**. *J Exp Zool* 1963, **154**:169–173.

48. Suga H, Tschopp P, Graziussi DF, Stierwald M, Schmid V, Gehring WJ: **Flexibly deployed Pax genes in eye development at the early evolution of animals demonstrated by studies on a hydrozoan jellyfish**. *Proc Natl Acad Sci USA* 2010, **107**:14263–14268.

49. Sun H, Dickinson DP, Costello J, Li WH: **Isolation of Cladonema Pax-B genes and studies of the DNA-binding properties of cnidarian Pax paired domains.** *Mol Biol Evol* 2001, **18**:1905–1918.

50. Taddei-Ferretti C, Musio C, Santillo S, Cotugno A: **The photobiology of hydra's periodic activity**. *Hydrobiologia* 2004:129–134.

51. Taddei-Ferretti C, Musio C: **Photobehaviour of hydra (Cnidaria, Hydrozoa) and correlated mechanisms: a case of extraocular photosensitivity.** *J Photochem Photobiol B, Biol* 2000, **55**:88–101.

52. Thurm U, Brinkmann M, Golz R, Holtmann M, Oliver D: **Mechanoreception and synaptic transmission of hydrozoan nematocytes**. *Hydrobiologia* 2004, **530/531**:97-105.

53. Westfall JA, Yamataka S, Enos PD: **Ultrastructural evidence of polarized synapses in the nerve net of hydra**. *J Cell Biol* 1971, **51**:318–323.

54. Wilson E: **The heliotropism of hydra**. *The American Naturalist* 1891, **25 (293)**: 413-433.

55. Wood RL: **The fine structure of the hypostome and mouth of hydra. II. Transmission electron microscopy**. *Cell Tissue Res* 1979, **199**:319–338.

Table 1

Condition	Staining
Whole	11 of 11
Regenerates	
0h	0 of 6
12h	2 of 10
24h	6 of 8
48h	0 of 9
Budding	
Stage 1	0 of 8
Stage 2	1 of 9
Stage 3	9 of 16
Stage 4	5 of 12

Fig. 1

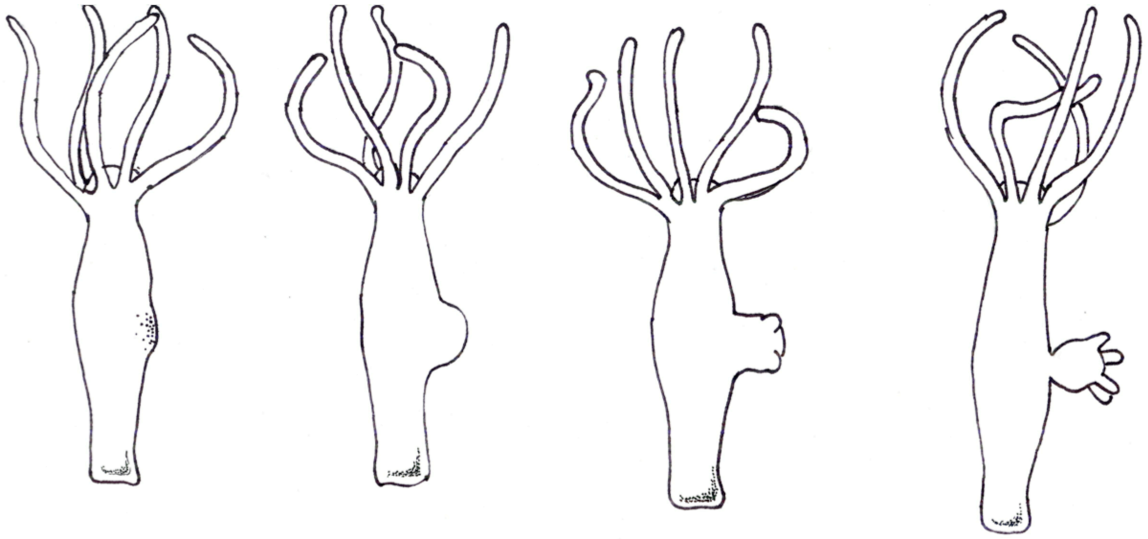


Fig. 2

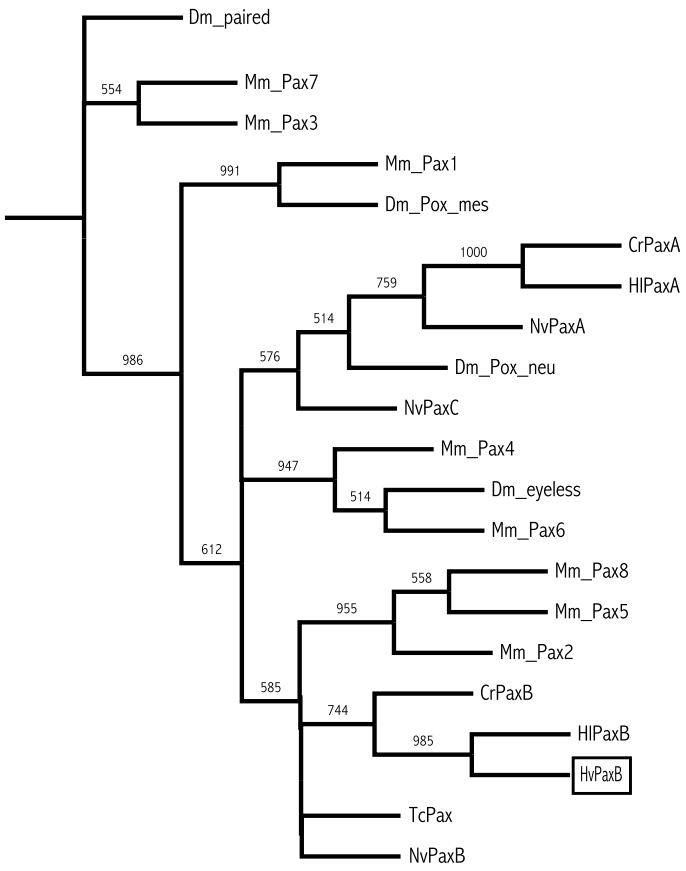

Dm_paired
554
Mm_Pax7
Mm_Pax3
991
Mm_Pax1
Dm_Pox_mes
1000
CrPaxA
759
HlPaxA
514
NvPaxA
986
576
Dm_Pox_neu
NvPaxC
Mm_Pax4
947
514
Dm_eyeless
Mm_Pax6
612
558
Mm_Pax8
955
Mm_Pax5
Mm_Pax2
585
CrPaxB
744
HlPaxB
985
HvPaxB
TcPax
NvPaxB

Fig. 3

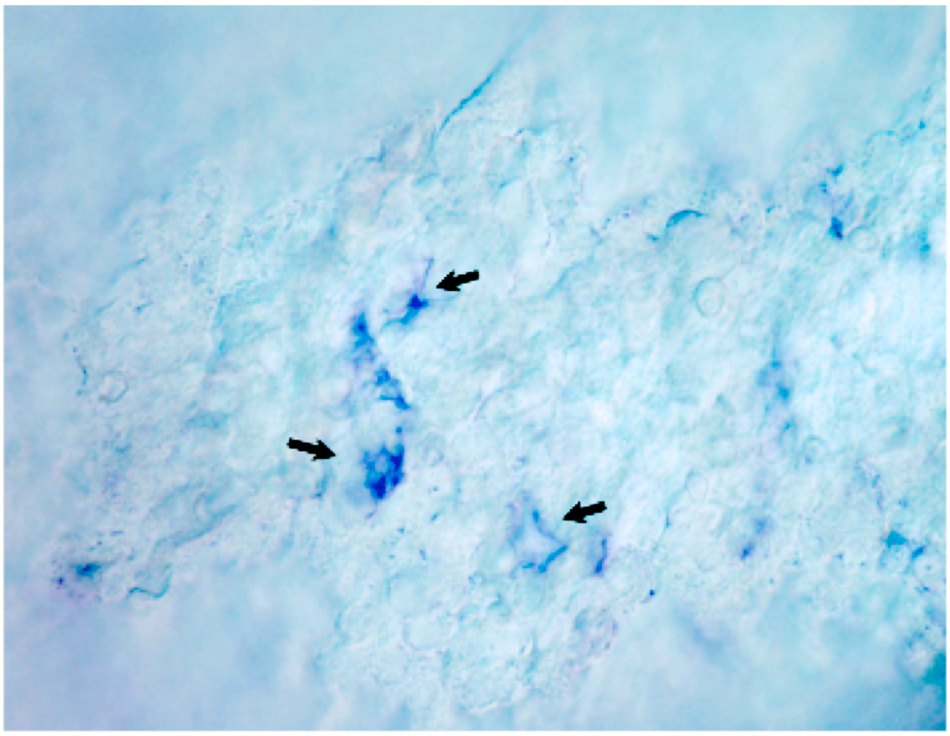


Fig. 4

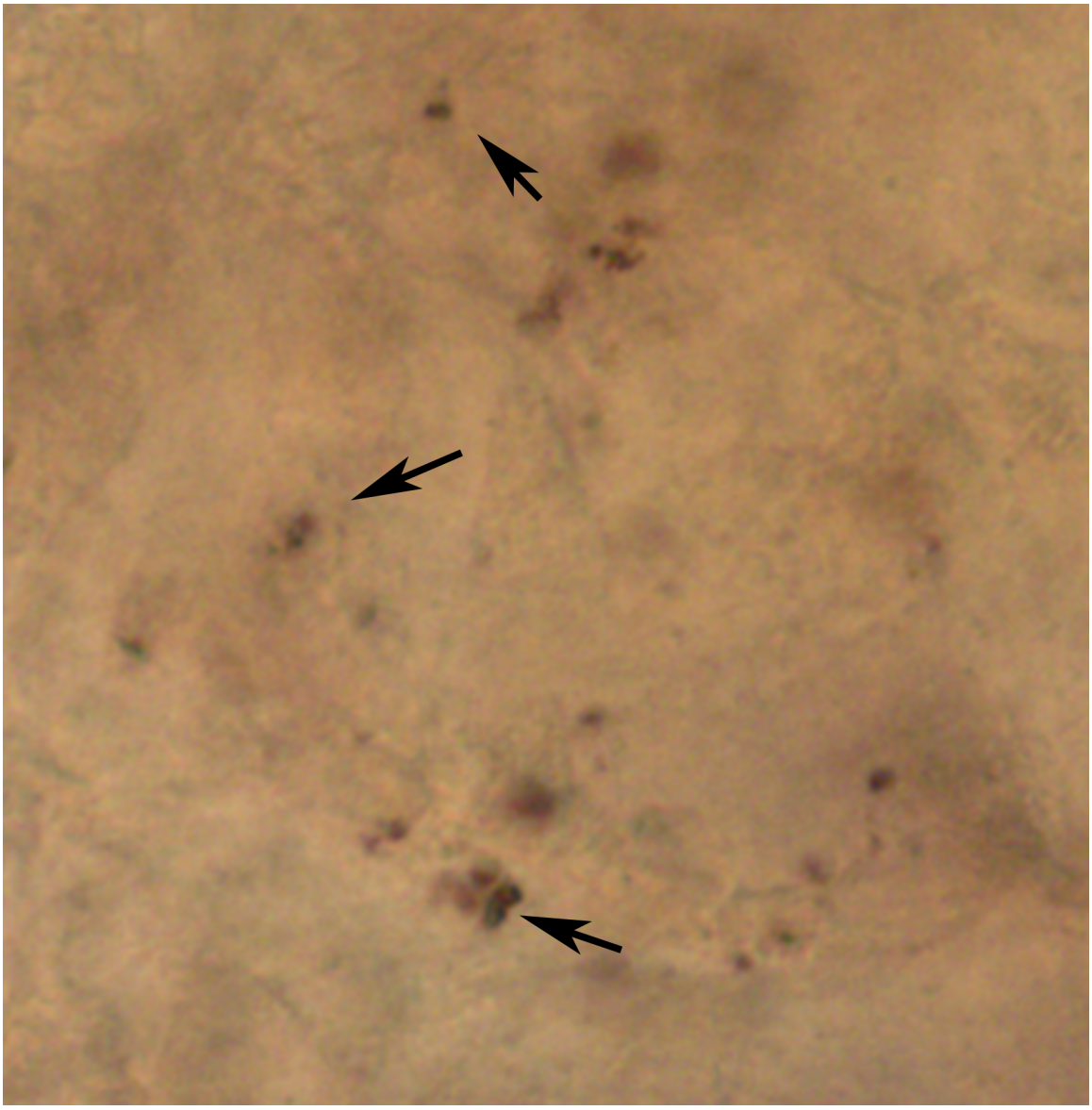


Fig. 5

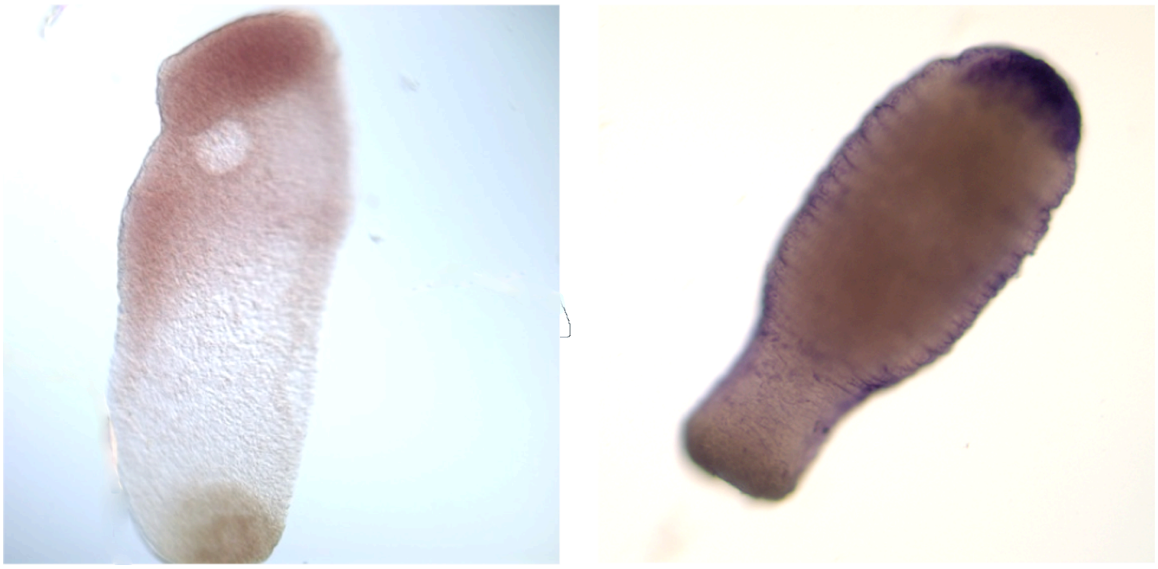


Fig. 6

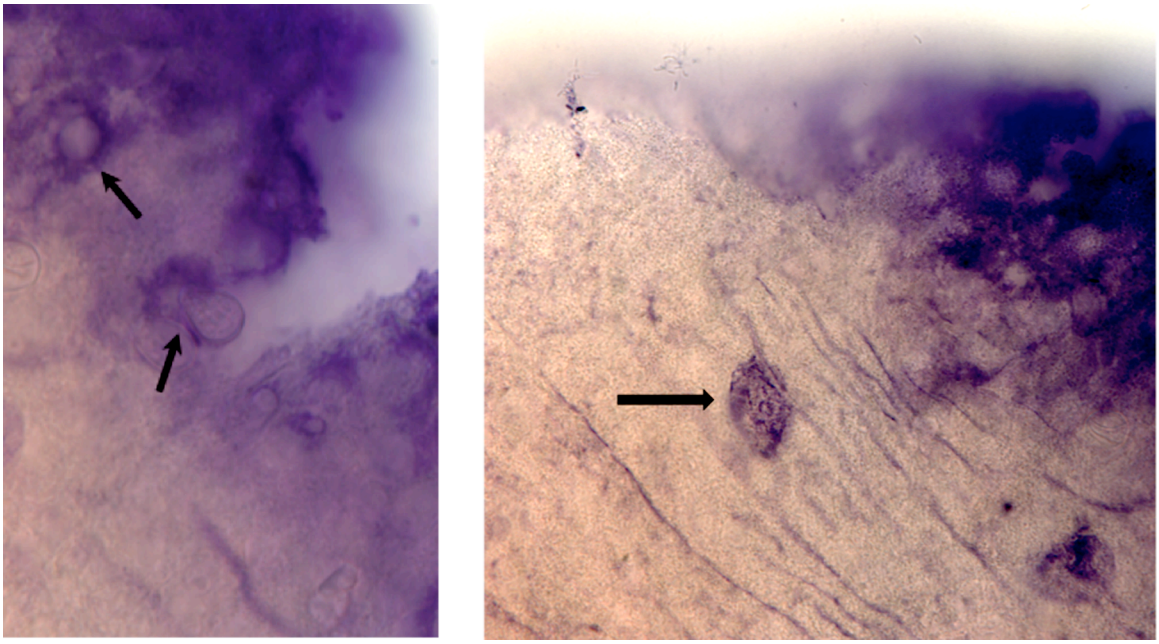


Fig. 7

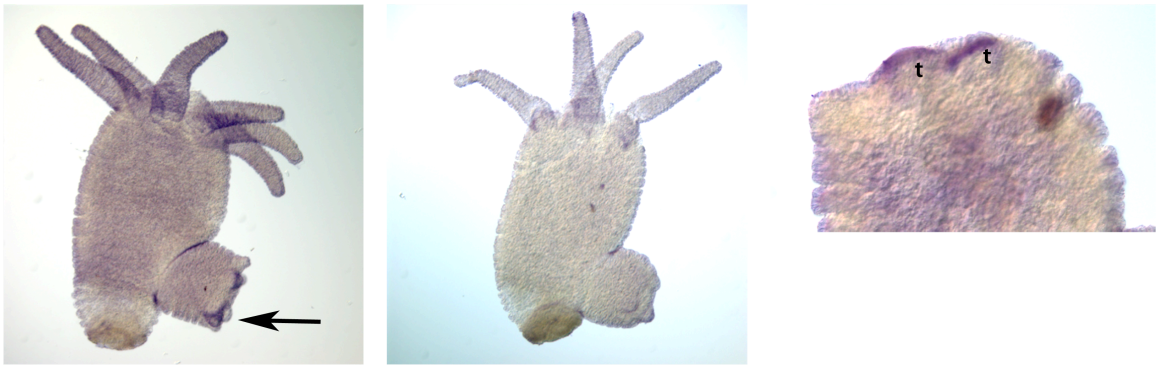


Fig. 8

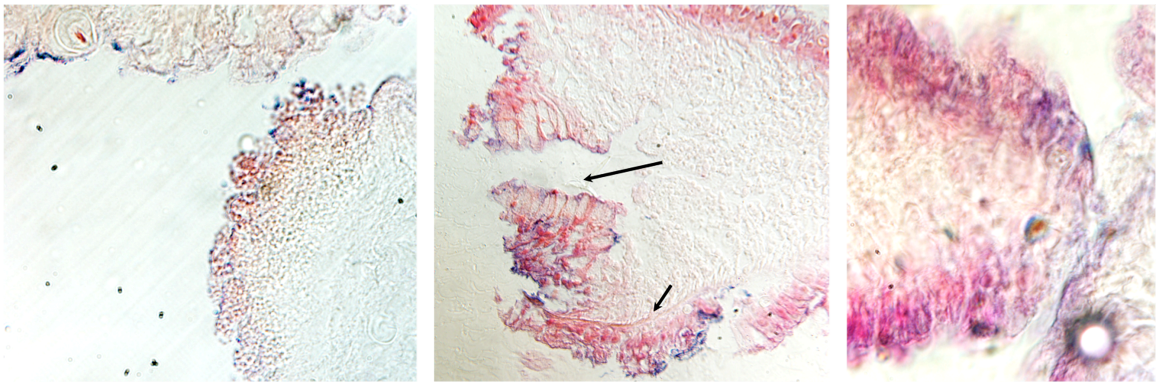
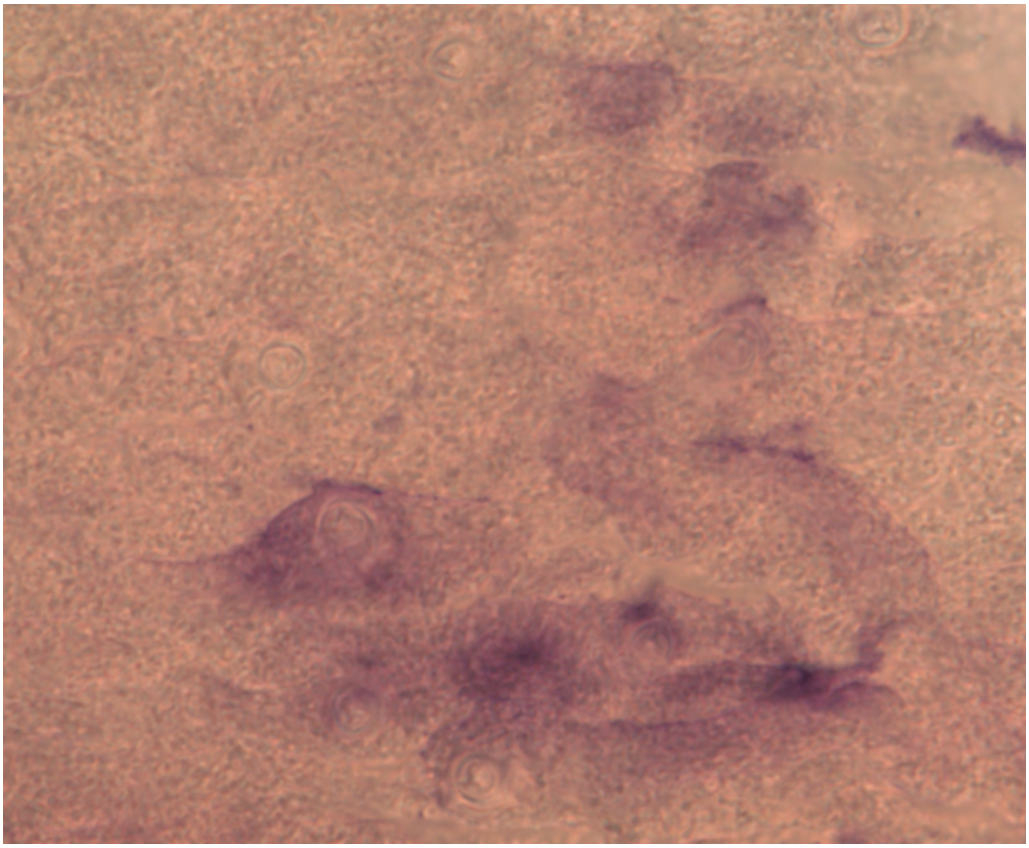


Fig. 9



Supplementary Fig. 1

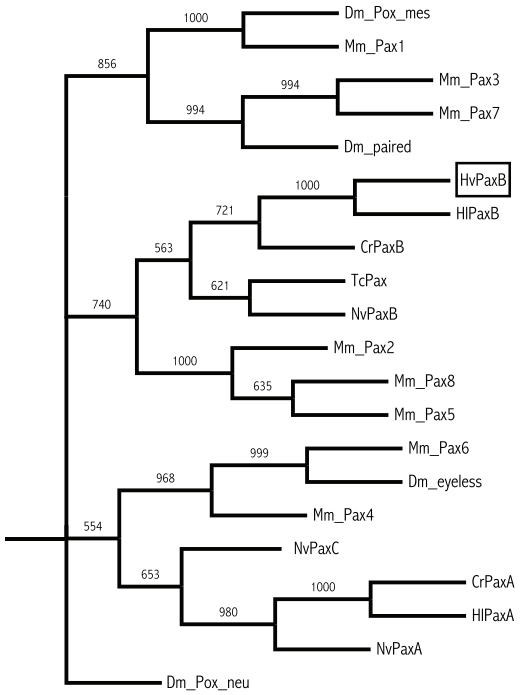
Pax gene sequence alignment

```
CrPaxA     YMRHRIIELAQCGVRPSEISRQLLVSHG
CrPaxB     PVRRKIVDLASQGVRPCDISRQLRVSHG
HlPaxA     YMRHRIIELAQCGVRPSEISRQLLVSHG
HlPaxB     PVRRKIVELAHQGVRPCDISRQLRVSHG
HvPaxB     SDPEFVIELAHQGVRPCDISRQLRVSHG
NvPaxA     YMRHRIVELAHCGVRPSEISRQLLVSHG
NvPaxB     VVRQRIVELAQSGVRPCDISRQLRVSHG
NvPaxC     YIRHRIIQLATYGVRPCEISRCLLVSHG
TcPax      QVRRRIVELAHQGVRPCDISRQLRVSHG
Dm_eyelessSTRQKIVELAHSGARPCDISRILQVSNG
Mm_Pax6    STRQKIVELAHSGARPCDISRILQVSNG
Mm_Pax2    VVRQRIVELAHQGVRPCDISRQLRVSHG
Mm_Pax4    DTRQQIVQLAIRGMRPCDISRSLKVSNG
Mm_Pax1    AIRLRIVELAQLGIRPCDISRQLRVSHG
Dm_Pox_mesATRMRIVELARLGIRPCDISRQLRVSHG
Dm_paired NIRLKIVEMAADGIRPCVISRQLRVSHG
Dm_Pox_neuCVRRRIVDLALCGVRPCDISRQLLVSHG
Mm_Pax3    HIRHKIVEMAHHGIRPCVISRQLRVSHG
Mm_Pax5    VVRQRIVELAHQGVRPCDISRQLRVSHG
Mm_Pax7    HIRHKIVEMAHHGIRPCVISRQLRVSHG
Mm_Pax8    VVRQRIVDLAHQGVRPCDISRQLRVSHG

CVSKILGRYYETGSVRPGAIGGSKPK-VATPKVVCRIVKLKEENPCMFAWEIRNSLLAEG
CVSKILGRFYETGSIRPGVIGGSKPK-VATPSVVQKIADYKAQNPTMFAWEIRECLINNN
CVSKILGRYYETGSVRPGAIGGSKPK-VATPKVVCRIVKLKEENPCMFAWEIRNSLLAEG
CVSKILSRFYETGSVRPGVIGGSKPK-VATPSVVAKIQEYKQHNPTMFAWEIRDKLLSEQ
CVSKILSRFYETGSVRPGVIGGSKPK-VATPSVVAKIQEYKQHNPTMFAWEIRDKLLSEQ
CVSKILGRYYETGSVRPGAIGGSKPK-VATPKVVSKILEYKDKNPCIFAWEIRNNLLADG
CVSKILCRFYETGSIKPGVIGGSKPK-VATGNVVTKIAEYKLANPTMFAWEIRDRLLSEG
CVSKILGRYYETGSIRPGSIGGSKPK-VATPPVVNKILQYKQQNPTIFAWEIRDRLVEEG
CVSKILGRYYETGSIKPGIIGGSKPK-VATPGVVSKIAEYKRANPTMFAWEIRDRLLQDS
CVSKILGRYYETGSIRPRAIGGSKPR-VATAEVVSKISQYKRECPSIFAWEIRDRLLQEN
CVSKILGRYYETGSIRPRAIGGSKPR-VATPEVVSKIAQYKRECPSIFAWEIRDRLLSEG
CVSKILGRYYETGSIKPGVIGGSKPK-VATPKVVDKIAEYKRQNPTMFAWEIRDRLLAEG
CVSKILGRYYRTGVLEPKCIGGSKPR-LATPAVVARIAQLKDEYPALFAWEIQHQLCTEG
CVSKILARYNETGSILPGAIGGSKPR-VTTPNVVKHIRDYKQGDPGIFAWEIRDRLLADG
CVSKILARYHETGSILPGAIGGSKPR-VTTPKVVNYIRELKQRDPGIFAWEIRDRLLSEG
CVSKILNRYQETGSIRPGVIGGSKPR-IATPEIENRIEEYKRSSPGMFSWEIREKLIREG
CVSKILTRFYETGSIRPGSIGGSKTKQVATPTVVKKIIRLKEENSGMFAWEIREQLQQQR
CVSKILCRYQETGSIRPGAIGGSKPKQVTTPDVEKKIEEYKRENPGMFSWEIRDKLLKDA
CVSKILGRYYETGSIKPGVIGGSKPK-VATPKVVEKIAEYKRQNPTMFAWEIRDRLLAER
CVSKILCRYQETGSIRPGAIGGSKPRQVATPDVEKKIEEYKRENPGMFSWEIRDRLLKDG
CVSKILGRYYETGSIRPGVIGGSKPK-VATPKVVEKIGDYKRQNPTMFAWEIRDRLLAEG

ICDNGNVPSVSSINRILRNHAAEKETKEA
ICDVESVPSVSSINRIVRNRIGGGGKVSK
ICDNGNVPSVSSINRILRNHAAEKETKEA
ICDSDSVPSVSSINRIVRNRLGSSSHASM
ICDSDSVPSVSSINRIVRNRLGPSSHASM
VCDKTNVPSVSSINRILRNSAAEKEARAV
VCTSDNVPSVSSINRIVRNRINSQDKMSN
ICDRDNTPSVSSINRILRNKAAERAAQFA
VCSQENVPSVSSINRIVRNRINSTGKEEE
VCTNDNIPSVSSINRVLRNLAAQKEQQST
VCTNDNIPSVSSINRVLRNLASEKQQMGA
ICDNDTVPSVSSINRIIRTKVQQPFHPTP
LCTQDKAPSVSSINRVLRALQEDQSLHWT
VCDKYNVPSVSSISRILRNKIGSLAQPGP
ICDKTNVPSVSSISRILRNKLGSLGHQHT
VCDRSTAPSVSAISRLVRGRDAPLDNDMS
VCDPSSVPSISSINRILRNSGLWTDEMTS
VCDRNTVPSVSSISRILRSKFGKGEEEEA
VCDNDTVPSVSSINRIIRTKVQQPPNQPV
HCDRSTVPSVSSISRVLRIKFGKKEDDEE
VCDNDTVPSVSSINRIIRTKVQQPFNLPM
```

Supplemental Fig. 2



CHAPTER 3

Effects of Light on Isolated Tentacles of Hydra vulgaris

Stephanie L. Guertin[2], and Gabriele Kass-Simon[1,2], *

[1]Department of Biological Sciences, College of the Environmental and Life Sciences, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881-0816

[2]Interdisciplinary Neurosciences Program, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881-0816

* Author for correspondence (email: gkass.simon@uri.edu)

In preparation for submission to:

Comparative Biochemistry and Physiology A: Physiology

Abstract:

Previous electrophysiological studies on the cnidarian *Hydra vulgaris* have shown that they have a highly developed and very specific photoresponse, despite their lack of any structure recognizable as a traditional photoreceptor. In this work, in an effort to isolate the cell types and pathways that may be responsible for the photobehavior, we recorded extracellularly from single excised tentacles. The tentacles were exposed to wavelengths of light from 450 nm to 600 nm, as well as red and white light. Exposure to light caused a change in response that varied by color, with the number of large tentacle pulses significantly increasing at 550 and 600 nm, and the number of small tentacle pulses trending downward in 500 nm light. In addition to the traditional contraction bursts, long trains of pulses were observed. A change in light condition caused a switch from bursting to train patterns of activity or vice versa. These results indicate that isolated tentacles can and do respond to more than just white light, and can distinguish between colors across the visible spectrum.

Introduction:

Hydra, early-evolved metazoans of the Cnidaria, are tubular cnidarians whose bodies consist of two epithelial layers, the endoderm and ectoderm, separated by an acellular middle layer, the mesoglea. A ring of five to eight tentacles surrounds a mouth. The animals attach to the substrate via adhesion of a basal disc at the aboral end of the body column. Hydras possess a complete ectodermal nerve net and have gap-junctional connections between the ectodermal and endodermal epithelial cells (Hufnagel and Kass-Simon 1976, Wood 1979). Histological evidence indicates that there are two nerve rings in the hypostome, one of which appears to be coincident with the physiologically defined ring that integrates and coordinates tentacle and body contractions (Hufnagel & Kass-Simon 1976, Kass-Simon 1973, Kinnamon 1981, Koizumi 1992, Rushforth 1971, 1973, Passano and McCullough 1962, 1964, 1965).

Within the tentacles is a cell type of particular interest to this study, the battery cell complex (Hufnagel 1985). The battery cell complex (BCC) is a single large epitheliomuscular cell which encloses and supports a complement of sensory and effector cells. These include sensory neurons, myonemes, and various types of nematocyte. Battery cells are hypothesized to be the basic functional unit of the tentacle, and are arranged into battery cell complex rings, a group of four or more battery cells which form a ring around the tentacle. In the more distal regions of the tentacle, the battery cell complexes are the only type of ectodermal cell. Their interdigitating myonemes and neuronal processes allow for conduction of impulses and contractions up and down the tentacle, as well as coordinated firing of the

nematocytes in response to stimulus.

Despite their relatively simple anatomical organization, hydra have a complex variety of sensory modalities. Of particular interest is their response to light. Hydra, unlike some other cnidarians, bear no eyes, eyespots, or other distinct photoreceptive structures. This extraocular photosensitivity may be the oldest extant type of photosensitivity, appearing in a variety of species from bacteria through vertebrates, and present in non-specialized cells, neurons, and loose aggregations of photopigment in cells (Arendt 2003). Though lacking in specific photoreceptive structures, hydra do have a variety of specialized sensory and effector cells, some of which may play a role in photosensitivity. Candidates for this include sensory neurons of the ectoderm, battery cells of the tentacle, and nematocytes in body and tentacle. Despite the responsible cell type remaining unknown, hydra have a well-developed light response that has been known since the 1890s (Wilson 1891). The most immediately obvious evidence of this is their frequently described 'somersaulting' behavior, which allows them to quickly move toward a light source (Ewer 1947, Feldman and Lenhoff, 1962)

In Wilson's early experiments, *Hydra pirardi* were exposed to different colors of light by means of panes of colored glass placed over their aquarium and quantified by the adjacent Fraunhofer lines (Wilson 1891). The Fraunhofer lines are a series of measured points corresponding to dark lines on the visible emission spectrum of the sun (supplemental figure 1). The animals were found to congregate preferentially under blue light. Haug (1933) found that hydra appeared unresponsive to red light altogether, with pacemaker activity that did not differ from that in ambient light.

Later work by Passano and McCullough showed that shining white light on a

narrow band at the foot of dark-adapted *Hydra vulgaris* caused first extension and then contraction (Passano and McCullough 1964). Additionally, in these whole animals, exposure to light of less than 500 nm produced a decrease in the frequency of contractions pulses of the endodermal musculature, resulting in body column extension, as well as an increase in the rate of rhythmic potentials. The contractile bursts were also susceptible to interruption by light, as a six-second burst of bright light would stop a current burst. Subsequent bursts would increase in number of pulses per burst after such an interruption, with the number of pulses per burst continuing to increase with every time that a burst was interrupted for a single animal.

In bodiless head and tentacle preparations, as well as headless bodies, exposure to pulses of colored light changed the frequency and timing of ectodermal contraction pulses (Taddei-Ferretti et al. 2004). In addition Taddei-Ferretti, et al describe a large slow electrical change in baseline which the authors refer to as "the big slow wave" and which does not seem to be related to the classical rhythmic pulses but is changed by exposure to light.

In other experiments, *H. pirardi* was shown to have the swiftest change in behavior patterns when exposed to 350 to 500 nanometer light, with responses decreasing markedly above 500 nm before returning slightly at above 575 nm. The contractile response was also affected by the intensity of the light, with the speed of the onset of the first contraction directly proportional to the intensity. Intensity of light was also shown to affect the tentacle contraction pulses in isolated tentacles, with pulses of strong white light causing an increase in tentacle contraction bursts compared to ambient light (Singer et al., 1963).

The results in light seen by Passano and McCullough, Taddei-Ferretti, et al. and Singer all give a picture of a very defined photoresponse, with the greatest increase in activity in high intensity light below a wavelength of 500 nm. Despite this clearly defined response, the anatomical diversity of the responding regions of the animal has made discernment of the specific cell types and pathways involved in the photoresponse unclear. Either this photosensitivity is found in many cells, or the cell types responsible are widely distributed.

A number of attempts to locate the genetic basis of this photosensitivity have also been made. Multiple opsin-like photopigment genes have been isolated from hydra genomic DNA using degenerate primers formed from the rhodopsin genes in twelve different invertebrates; the finding of genes for multiple photopigments hypothesized to respond to different wavelengths is consistent with the suggestion that hydra are capable of discerning colors (Santillo et al. 2006). Additionally, in hydra, one such opsin has been shown to colocalize with the expression of genes for cyclic nucleotide-gated (CNG) proteins, like those found in the phototransduction cascade in other taxa (Plachetzki et al. 2007). Inactivation of the CNG channels resulted in a loss of firing of nematocysts when the animal was exposed to light (Plachetzki et al. 2012).

Although isolated tentacles have been found to respond to specific high intensity light stimulation, and because other experiments indicate light sensitivity in other regions of the body, in order to specifically characterize the tentacular light response, we removed the tentacles, allowed them to heal, and recorded extracellularly from the base of each tentacle. This allowed the responses of the

tentacles to be examined when isolated from the nerve network of the hypostome. Not only would this allow for examination of the responses of the tentacles to light, but also a comparison of the response patterns of isolated tentacles to that known in other preparations in which the hypostome is present would indicate that transmission and modification of the behavioral response is occurring after the reception of the light signal. In hypostome-free preparations, at least some of this modification is lost.

Materials and Methods:

Animal culture:

Specimens *of Hydra vulgaris* were asexually cultured in glass baking dishes containing modified bicarbonate versene culture solution (BVC): 1 x 10 - 7 mol/L NaHCO3, 1 x 10-6 mol/L CaCl2, and 1 x 10-8 mol/L EDTA, pH 7.0 +/- 0.2 (Loomis and Lenhoff 1956, Muscatine and Lenhoff 1965). All animals were kept in darkness in an incubator at 18 +/- 1.0 °C. Hydra cultures were fed freshly hatched *Artemia salina* nauplii once every 48 h.

Experimental Preparations:

Twenty-four hour starved animals were placed in BVC in a petri dish under a light microscope and allowed to relax. At maximal extension, the tentacles were cut off with a scalpel, ensuring that the cut was sufficiently below the insertion of the tentacles so that the putative nerve ring and site of contraction burst pacemaker origin (Hufnagel and Kass-Simon, unpublished; Kass-Simon, 1972) in the hypostome at the base of the tentacles remained intact and no hypostomal tissue carried along with the tentacle. Thus, after the ablation of a tentacle, the hypostome continued to bear a tentacle stub of approximately 0.5 mm, out of a total tentacle length of approximately 7 mm (Fig. 1). The tentacles were then placed in fresh BVC and allowed to heal for 6 hours. Once healed, the tentacles were prodded with a pipette to ensure they were still alive and capable of contraction. The tentacles were then allowed to relax again in the dark for 5 minutes to eliminate any residual effects of this testing.

Electrophysiology:

A single tentacle was attached to a suction electrode in BVC under a dissecting microscope (Fig. 2). The experimental protocol was as follows: ten minutes of darkness, ten minutes of the tested light wavelength, ten minutes of white light. Colored light was provided with the use of narrow bandpass (50 nm) filters at 450, 500, 550, and 600 nanometers (Edmund Optics). All wavelengths were adjusted to a constant intensity of 1100 lux using neutral density filters (Edmund Optics) and intensity measured using a Luna-Pro lightmeter. All electrical recordings were made at 22.0 C +/- 2.0 C, in 5 mL dishes filled with 3 mL of BVC, in complete darkness. Impulses were led into the head stage of an A-M Systems Model 3000 amplifier and then into an AD Instruments PowerLab. The digital output was recorded with LabChart 7 on a MacBook Pro. The protocol was essentially that of Kay and Kass-Simon, 2009 and Ruggeri, et al., 2004. Impulses, characterized by their shape and size, were then measured during each of the three periods and compared using FANOVAs and the appropriate post-hoc tests (MATLAB *friedman* and *multcompare)*.

Tentacle Pulses

In the tentacles, two types of pulses were recorded: large tentacle pulses (LTPs) and small tentacle pulses (STPs), a subset of which were rhythmic potentials (RPs). Large tentacle pulses were larger in size, even greater than 1mV. Small tentacle pulses ranged from 3 to 10μV in size, with a recognizable shape that distinguished them from the noise. Rhythmic potentials were a subset of these small pulses that occurred in groups with a recognizable periodicity and associated with an elongation

of the tentacle (Fig. 5).

Several overarching patterns of pulses were observed. Contraction impulses often arise in bursts of several pulses close together in time; the number of pulses per burst and the number of bursts per time were compared. A burst was defined as between four and fifteen pulses arising within 15 s of each other and followed by a period of silence of at least five seconds (Fig. 3). Shown is a burst of tentacle contraction pulses.

In some animals and conditions, groups of pulses arose that differed from the classical burst by a significant length. These groups of pulses, which we are calling trains, consisted of strings of pulses greater than a minute's duration and with more than fifteen pulses (Fig. 4, 6, 7).

Data Analysis:

For each of the seven tentacles used for each experimental condition, pulses were visually identified and sorted, and their amplitudes recorded. These data were then processed in Matlab and compared using FANOVAs via the matlab *friedman* command, with an alpha level of 0.05 and the appropriate follow-up test to determine which of the wavelengths produced impulses that were significantly different from baseline using *multcompare*. Significance was defined as $p \leq 0.05$, with $0.05 < p \leq 0.1$ interpreted as a trend.

Results:

Large Tentacle Pulses: In isolated tentacles, there are two principal spiking patterns, essentially uncoupled from the visible movement of the tentacle itself. The first, previously mentioned, is the bursting pattern characterized by the large tentacle pulses coming in groups of four or more with an extended period of silence afterward. When light conditions changed, the number of large tentacle pulses (LTPs) showed significant change from baseline, with the direction of the change varying by tested wavelength (p=.004 using FANOVAs) - figure 8 and table 1. The largest increase in activity was in the higher wavelengths, most particularly 550 and 600 nm light (p = 0.329). In 550 nm, the median number of pulses was 109 pulses ± 71; in 600 nm light, the median number of pulses was 104 pulses ± 74). Exposure to 450 nm light resulted in a decreasing trend in the number of these same large pulses relative to white light, though this effect fell beneath our significance threshold (median 26 pulses ± 25.5, p = 0.0931). Both white and red light produced a slightly increased pulse rate.

Small Tentacle Pulses: Distinct from the LTPs were the small tentacle pulses, or STPs, a subset of which were the rhythmic potentials. Neither STPs or RPs showed a significant change relative to changing light conditions when compared across all colors at P = 0.05. However, at P = 0.0939, a trend was evident in the STPs (Fig. 9).

The rhythmic potential system of the tentacles also did not show a clear and significant response to different wavelengths of light across the spectrum, although a trend toward change was observable (p = 0.0941). The sole exception to this was at

500 nm, where exposure to light resulted in a significant decrease in activity relative to light, with a median of 11 RPs ± 4 ($p = 0.0415$).

Pulse Trains: We have characterized the second behavior as a pulse train. In these trains, the large tentacle pulses occur as a series of impulses that continue past the usual 15-pulse length of the burst, typically followed by an extended period of silence (Fig. 4). The duration of these trains is 1 to 10 minutes, compared to bursts, which usually last less than a minute. Most trains lasted five minutes or more and included fifty or more single pulses, generally at a slower rate than those found in bursts. In most animals that displayed the train behavior, a change in the light condition from darkness to any wavelength of light was sufficient to produce a change in behavior pattern (Table 3).

Additionally, the interpulse interval may change repeatedly during a train, increasing and decreasing in a repetitive pattern similar in appearance to many bursts superimposed upon each other. A change in the light condition, in many animals, caused a switch from a bursting pattern of activity to a train, or the reverse. The large tentacle pulses often arose in different locations, as seen in the changed polarity, though not absolute amplitude or shape, over time (Fig. 11).

***Effects of light on tentacle contraction bursts*:**

Light produced a change in the number of contraction bursts recorded, although the number of pulses per bursts did not change and did not differ from that found in other preparations (Table 1). The greatest change occurred when the tentacle was

exposed to 550 nm light, showing a marked increase in the rate of tentacle contraction bursts (median 5 bursts ± 3, p = 0.0398). The effect in 600 nm light was similarly trending upward (median 5 bursts ± 1.5, p = 0.0739).

It was found that the bursting rate in all colors of light differed significantly from the bursting pattern in darkness (p=.0239). The strongest effect was seen in 450 and 500 nm light, with 450 nm light producing a significant decrease in the rate of bursting (-1 burst ± 4, p = 0.0206), and 500 nm light (4 bursts ± 2, p = 0.419) producing the strongest increase in bursts over time.

Impulses in white light and darkness:

Continued exposure to darkness resulted in a continued diminution of the spike amplitudes. When all spikes are plotted against time (Fig. 10), this is evident in the increasing proportion of the spikes falling in the smaller ranges. Continued exposure to white light does not show this diminution over the course of the stimulus. Before 600 seconds into the recording, the initial exposure to white light, the maximal spike amplitude decreases by as much as 64% over the seven preparations tested. During exposure to white light, the maximum decrease in spike amplitude across this twenty minute period is 19%.

Discussion:

In summary, we have confirmed that excised hydra tentacles are sensitive to light, even in the absence of the head and body. Moreover, the behavior of the tentacles changes in measurable ways depending on the wavelength of the light to which the tentacle is exposed.

We first wish to deal with the new evidence that hydra are not insensitive to red light. Previous studies of hydra have indicated that hydra were incited to contraction by pulses of white light, but that they were blind to red (Wilson 1891). Further work described the output of the head pacemaker in red light as is similar to that in darkness (Passano and McCullough 1962, 1964). Our results here show that the response to red light, while not as dramatic as that to blue and green light, is still present in isolated tentacles. It may be that earlier findings indicating that hydra do not respond to red light are true only for the behavior of the animal when including the head and tentacles, perhaps suggesting that there is a form of post-processing active at the head that receives and subsequently dismisses the inputs from red light. Given the rapid attenuation of red light in water, it may be that this is a response to the environment, as it would be energetically expensive to maintain a photoreception mechanism specific to a wavelength of light rarely found.

Secondly, the most significant changes in activity were seen in the blue and green wavelengths (450 to 550 nm). Conversely to the rapid attenuation of red light in water, these wavelengths carry furthest. Hydra's increased contractile activity in blue and green light may relate to feeding behavior, the stimulus caused by the movement of prey animals through the water, and the changes in light caused by small,

semitransparent organisms.

Our most interesting findings relate to the changes in activity patterns with changes in light conditions. Tentacle pulses in isolated tentacles display two basic patterns of activity, as outlined previously - the burst and the train (Fig. 4, 6, 7). Since trains frequently arise from bursts, it is possible to consider that a train is a burst extended long past the normal termination point. The bursts show more than one of the classical patterns of bursting: steady rates, increasing frequencies, and decreasing frequencies of impulses were all observed. A change in light often provoked a shift in the type of response. Whereas Passano and McCullough (1964) found that when contraction bursts were repeatedly blocked by light, the frequency of bursts thereafter was increased, in tentacles this was not found. Rather, a change in illumination caused a shift in overall behavior pattern from bursts to trains or vice versa. A burst or train already in progress was not interrupted. It appears that at least some of the pacemakers responsible for the bursting behavior associated with the tentacle contraction pulses are located in the head and not present in single-tentacle preparations. Some of the tentacle pulse pacemakers must, however, be endogenous to the tentacles, as all bursting behavior is not lost.

Previous work has located ganglia at the bases of the tentacles, involved with the circumferential nerve ring and the tentacular nerve net (Hufnagel et al 1985). It was surmised that these ganglia are responsible for the coordination of the tentacle pulses. Additionally, a string of tentacle pulses have been shown to increase in amplitude until they trigger a through-conducted body contraction, in a process known as facilitation (Passano and McCullough 1963, Kass-Simon 1970, 1972,

Rushforth 1971). This process may also include feedback to the tentacle to terminate the tentacle pulse burst; a loss of this feedback with the removal of the head may play a role in the production of the long trains of pulses seen in isolated tentacles. The addition of light seems at least partially able to substitute for the head's feedback.

In darkness, long pulse trains and bursts of gradually decreasing spike amplitude may continue for long periods. Exposure to light appears to cause a more steady-state response, with the large tentacle pulses becoming more uniform in size. We hypothesize that the continued light stimulus prevents the diminution in the dark.

Previous experiments indicate that tentacle pacemaker impulses feed into the hypostomal pacemaker system. In these headless preparations, we theorize that these trains are due to a loss of inhibitory inputs from the head that would normally end the spiking behavior. This correlates with extended spike trains like this when the hydra were exposed to atropine (Kass-Simon, 1978). As with the trains observed after exposure to atropine, the amplitude of pulses was reduced by the end of a spike train.

Burst Patterns

The tentacle pulse bursts observed (Fig 3 and 5) are similar to parabolic bursting patterns described in other organisms, including the sea cucumber *Aplysia,* with a change in rate over the course of the burst (Strumwasser 1967). The degree of the parabolic nature of the burst differs from burst to burst. Bursting neurons have previously been separated into Type I, Type II, and Type III patterns in this manner; in single neurons, it is thought that one type of stimulus in one type of neuron will consistently produce one type of burst (Bertram et al. 1995, Rinzel et al. 1987). The

presence of multiple patterns of burst activity in our recordings may indicate that these pulses do not originate from single neurons across the length of the burst; instead, the output of many neurons together creates each burst and causes a changing frequency pattern from burst to burst.

In previous studies, light was shown to affect the timing and pattern of bursting neurons and groups of neurons (Strumwasser 1967, Taddei-Ferretti 2004). Particularly of interest here was the work done in *Aplysia* eyes by Strumwasser, which showed that light could affect the entrainment of bursts in the eye. Our results here show a pattern of pulses (Fig. 11) of similar amplitude and shape, but different polarity. These pulses are correlated with contractile activity of the tentacle, and, particularly when they occur in trains, these pulses appear to originate in a number of different locations around and along the length of the tentacle. In these bursts and trains, the visible electrical response is the aggregate response from many excitable cells in the tentacle. Since a change in light conditions results in a change in pattern, the change could be due to the excitement of a new locus, thus setting off a change in pattern. This is similar to the shift in excitation locus described by Passano and McCullough (1964), in which a change in the light condition in whole animals could shift the behavior pattern both spatially and temporally.

To an electrode placed at the base of the tentacle, this would give the appearance seen in the tentacle pulses. Combined with previous findings on the organization of the tentacle nervous networks and the role of the nematocytes and battery cells in light reception, it is possible to hypothesize that they serve here as the origination points for the tentacle pulses. Possible candidates for this, then, include

the neurons forming a network down the tentacle itself, or possibly the battery cells (Hufnagel et al. 1985). The battery cells are known to contain a diverse array of sensory neurons in conjunction with nematocytes; these sensory cells affect the rate of firing of the nematocytes, responding to chemical and mechanical stimuli. When coupled with the findings from Plachetzki et al. (2012) that deactivation of the CNG channels results in reduced nematocyst response to light, it may be surmised that the battery cells include light reception and response in their suite of capabilities.

References:

Arendt, D. 2003. Evolution of eyes and photoreceptor cell types. *Int. J. Dev. Biol*, 47: 563-571.

Bertram, R., Butte, M.J., Kiemel, T., Sherman, A. 1995. Topological and Phenomenological Classification of Bursting Oscillations. *Bull Math Bio*. 57 (3): 413-439.

Feldman, M, Lenhoff, H.M. 1960. Phototaxis in Hydra littoralis: rate studies and localisation of the "photoreceptor." *Anat. Rec*. 137: 354-355.

Haug, G. 1933. Die Lichtreaktionen der Hydren (Chlorohydra viridissima und Pelmatohydra oligactis). *Z. Vgl. Physiol*. 19: 246–303.

Hufnagel, L., Kass-Simon, G. The ultrastructural basis for the electrical coordination between epithelia of hydra. *Coelenterate Ecology and Behavior: Selected Papers* (1976).

Kass-Simon, G. 1970. Multiple excitation sites and straight-line conduction in the contraction burst system of Hydra. *Am. Zool*. 10: 505. (Abstract).

Kass-Simon, G. 1972. Longitudinal conduction and contraction burst pulses from hypostomal excitation loci in Hydra attenuata. *J. Comp. Physiol*. 80: 29–49.

Kass-Simon, G. 1973. Transmitting systems in Hydra. *Publ. Seto Mar. Biol. Lab*. 20: 583–593.

Kass-Simon, G., Passano, L. 1978. A neuropharmacological analysis of the pacemakers and conducting tissues of Hydra attenuata. *J. Comp. Physiol. A,* 128, 71-79.

Kass-Simon, G., Pierobon, P. 2007. Cnidarian neurochemical transmission: an updated overview. *Comp. Biochem. Physiol. A* 146: 9 –25.

Kass-Simon, G., Scappaticci, A.A. 2002. The behavioral and developmental physiology of nematocysts. *Can. J. Zool.* 80: 1772– 1794.

Kinnamon, J. C., Westfall, J.A. 1981. A three dimensional serial reconstruction of neuronal distribution in the hypostome of Hydra. *J. Morphol.* 168: 321–329.

Koizumi, O., M. Itazawa, H. Mizumoto, S. Minobe, L. Javois, C. J. P. Grimmelikhuijzen, Bode, H.R. 1992. Nerve ring of the hypostome in Hydra. I. Its structure and maintenance. *J. Comp. Neurol.* 326: 7–21.

Loomis, W. F., Lenhoff, H.M. 1956. Growth and sexual differentiation of *Hydra* in mass culture. *J. Exp. Zool.* 132: 555–573.

Muscatine, L., Lenhoff, H.M. 1965. Symbiosis of hydra and algae. II. Effects of limited food and starvation on growth of symbiotic and aposymbiotic hydra. *Biol. Bull.* 129: 316–328.

Passano, L. M., McCullough, C.B. 1962. Light response and the rhythmic potentials of *Hydra. Proc. Natl. Acad. Sci. USA* 48: 1376– 1382.

Passano, L. M., McCullough, C.B. 1963. Pacemaker hierarchies controlling behavior

of *Hydra*. *Nature* 199: 1174–1175.

Passano, L. M., McCullough, C.B. 1964. Coordinating systems and behavior in *Hydra*. I. Pacemaker system of the periodic contractions. *J. Exp. Biol*. 41: 643–644.

Passano, L. M., McCullough, C.B. 1965. Coordinating systems in *Hydra:* the rhythmic potential system. *J. Exp. Biol*. 42: 205–231.

Plachetzki, D., Degnan, B., Oakley, T.H. 2007. The Origins of Novel Protein Interactions during Animal Opsin Evolution. *PLoS ONE*, 2 (10), e1054.

Plachetzki D.C., Fong C.R., Oakley T.H. 2012. Cnidocyte discharge is regulated by light and opsin-mediated phototransduction. *BMC Biol*. 10:17.

Rinzel, J. 1987. A formal classification of bursting mechanisms in excitable systems. In Mathematical Topics in Population Biology, Morphogenesis and Neurosciences, E. Teramoto and M. Yamaguti (Eds), Lecture Notes in Biomathematics, Vol. 71, pp. 267-281. Berlin: Springer.

Ruggieri, R. D., P. Pierobon, Kass-Simon, G. 2004. Pacemaker activity in *Hydra* is modulated by glycine receptor ligands. *Comp. Biochem. Physiol. A* 138: 193–202.

Rushforth, N. B., Burke, D. S. 1971. Behavioral and electrophysiological studies of *Hydra*. II. Pacemaker activity of isolated tentacles. *Biol. Bull*. 140: 502–519.

Singer R.H., Rushforth N.B., Burnett A.L. 1963. The photodynamic action of light on Hydra. *J Exp Zool*. 154:169–173.

Strumwasser, F. 1967. Types of information stored in single neurons. In Invertebrate Nervous Systems: Their Significance for Mammalian Neurophysiology, C. A. G. Wiersma (Ed.), pp. 290-319. Chicago: The University of Chicago Press.

Taddei-Ferretti C., Musio C., Santillo S., Cotugno A. 2004. The photobiology of hydra's periodic activity. *Hydrobiologia*. 129–134.

Taddei-Ferretti C., Musio C. 2000. Photobehaviour of hydra (Cnidaria, Hydrozoa) and correlated mechanisms: a case of extraocular photosensitivity. *J Photochem Photobiol B, Biol*. 55:88–101.

Thurm U., Brinkmann M., Golz R., Holtmann M., Oliver D. 2004. Mechanoreception and synaptic transmission of hydrozoan nematocytes. *Hydrobiologia*. 530/531:97-105.

Wilson, E. B. 1891. The heliotropism of hydra. *Am. Nat*. 25: 413–433.

Table 1

Tentacle Pulses

Wavelength		Mean and SD	Median and IQR
450 nm: Blue (n=7)	LTPs	52.43 ± 73.6	26 ± 25.5
	STPs	27 ± 33.45	13 ± 36.5
500 nm: Green (n=7)	LTPs	6 ± 7.09	3 ± 6.5
	STPs	5.14 ± 7.71	4 ± 4
550 nm: Yellow (n=7)	LTPs	82.71 ± 48.29	109 ± 71
	STPs	12.28 ± 5.76	11 ± 4
600 nm: Orange (n=7)	LTPs	93.14 ± 41.48	104 ± 74
	STPs	17.14 ± 21.08	6 ± 14.5
650 nm: Red (n=7)	LTPs	83.29 ± 67.51	59 ± 104
	STPs	24.71 ± 35.95	9 ± 19.5
White (n=7)	LTPs	68.71 ± 37.18	71 ± 29.5
	STPs	41.28 ± 18.27	43 ± 13.5

Table 2

Tentacle Contraction Bursts

Wavelength		Mean and SD	Median and IQR
450 nm: Blue (n=7)	Bursts	4.85 ± 5.24	4 ± 4.5
	Change from Dark	-1.43 ± 3.21	-1 ± 4
500 nm: Green (n=7)	Bursts	5.14 ± 1.36	5 ± 1.5
	Change from Dark	4 ± 1.41	4 ± 2
550 nm: Yellow (n=7)	Bursts	5 ± 2.71	5 ± 3
	Change from Dark	2 ± 1.73	2 ± 3
600 nm: Orange (n=7)	Bursts	5.14 ± 1.34	5 ± 1.5
	Change from Dark	1.57 ± 1.98	1 ± 2
650 nm: Red (n=7)	Bursts	3.57 ± 1.71	4 ± 2
	Change from Dark	0.57 ± 1.90	1 ± 1.5
White (n=7)	Bursts	3.71 ± 1.60	4 ± 1.5
	Change from Dark	1.29 ± 2.28	1 ± 3

Table 3

Tentacle Patterns

Wavelength	Animals Showing Trains	Pattern Changed Under Light
450 nm: Blue (n=7)	3	2
500 nm: Green (n=7)	3	3
550 nm: Yellow (n=7)	4	2
600 nm: Orange (n=7)	7	4
650 nm: Red (n=7)	5	5
White (n=7)	7	7

Fig. 1

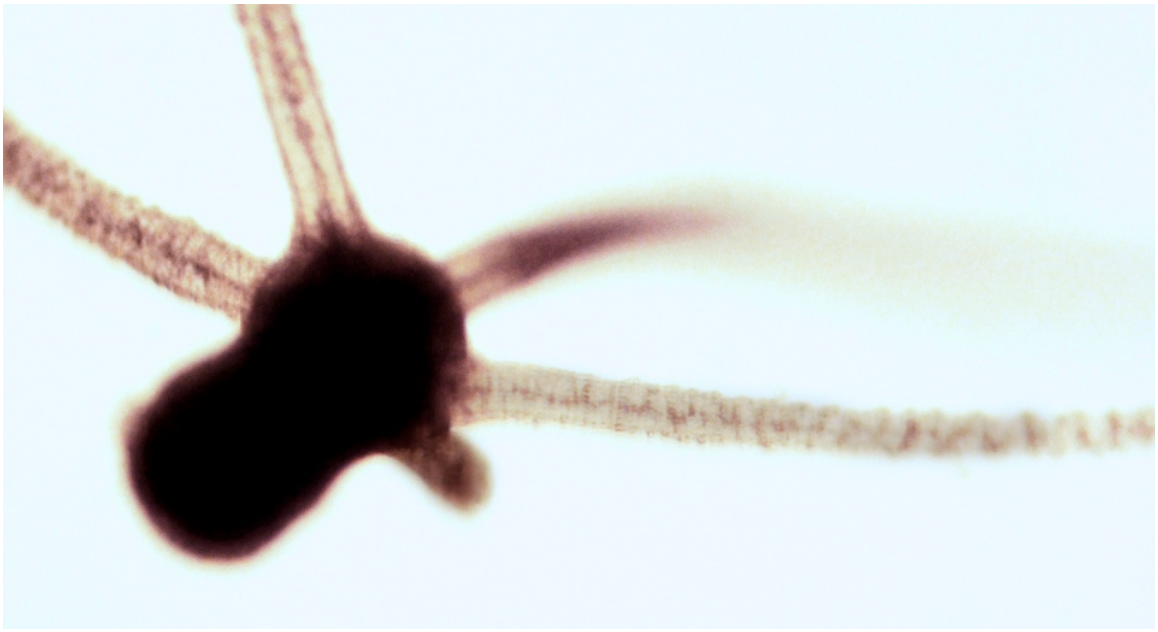


Fig. 2

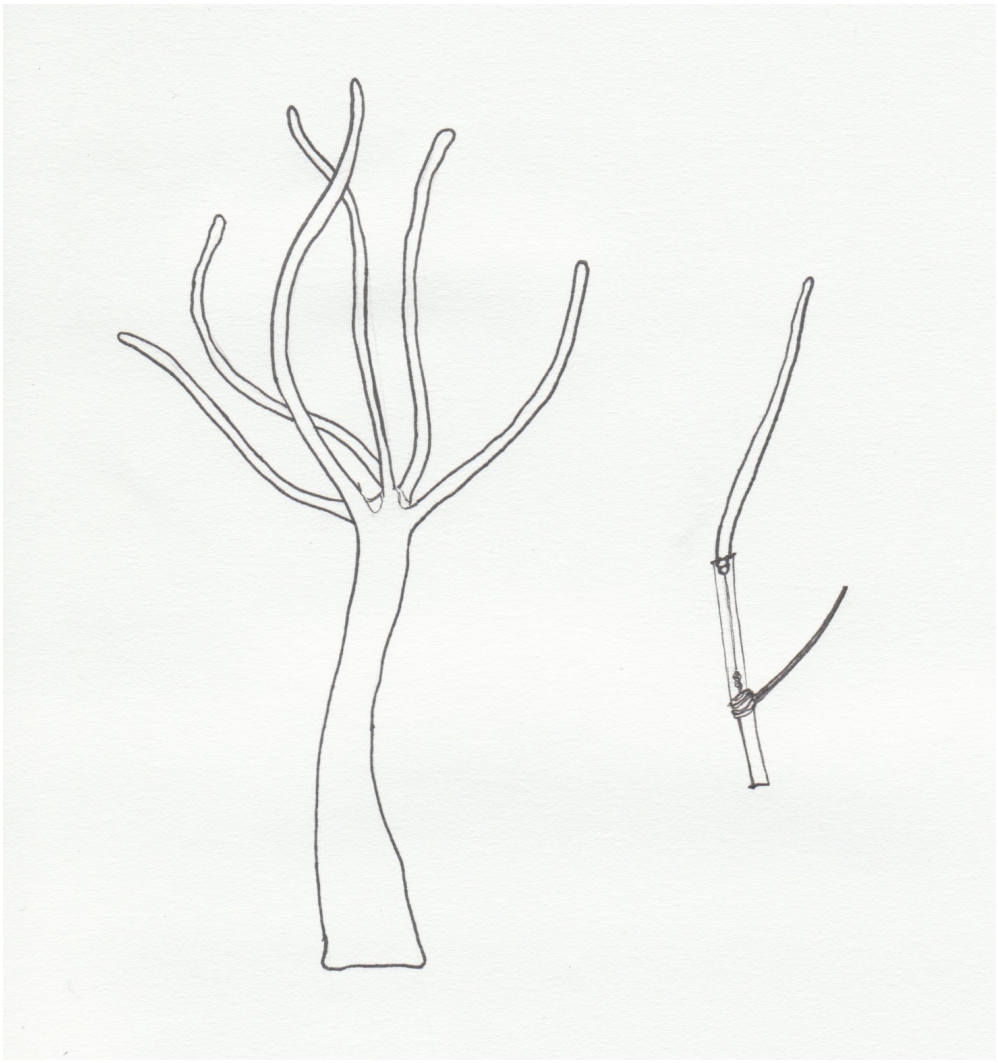


Fig. 3

Fig. 4

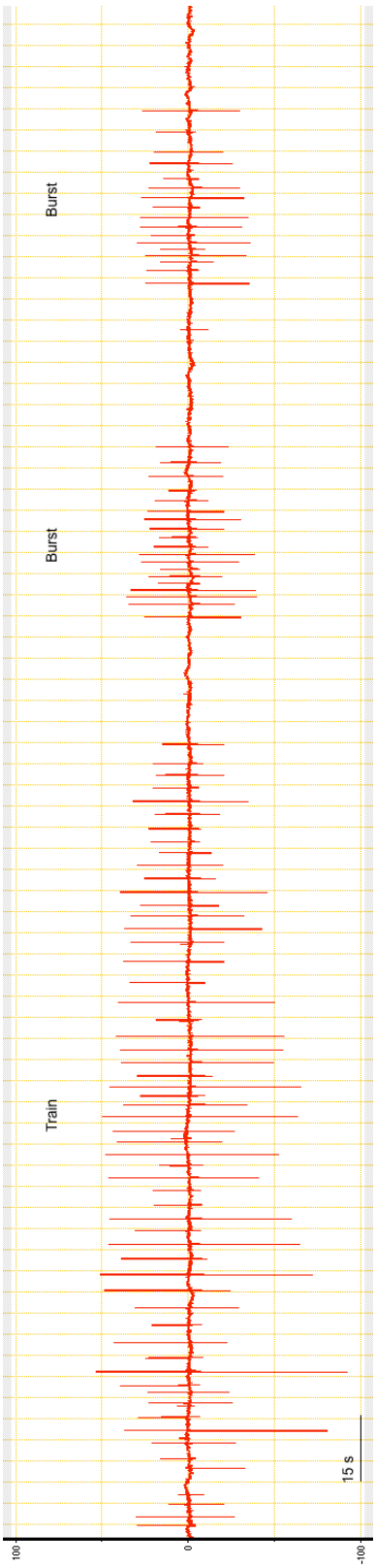


Fig. 5

Fig. 6

Fig. 7

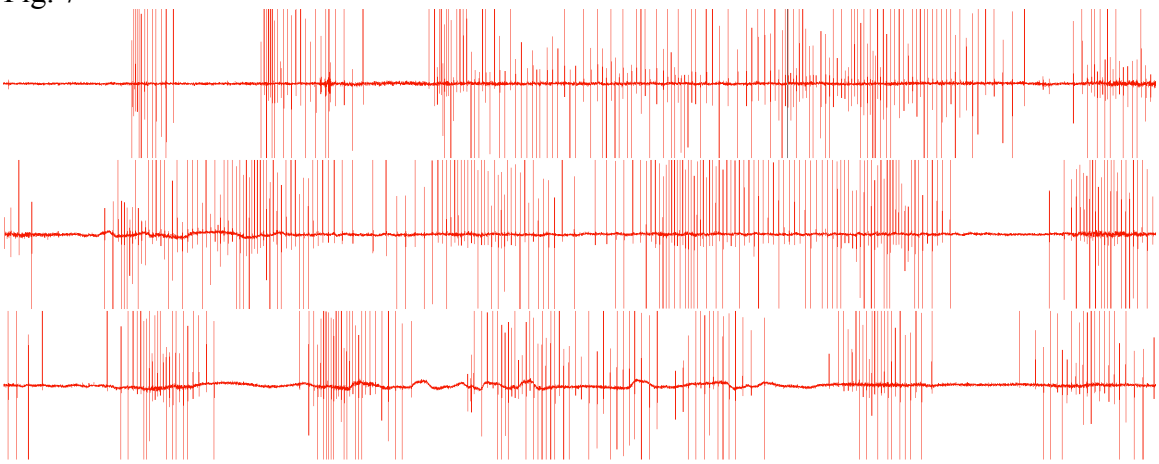


Fig. 8

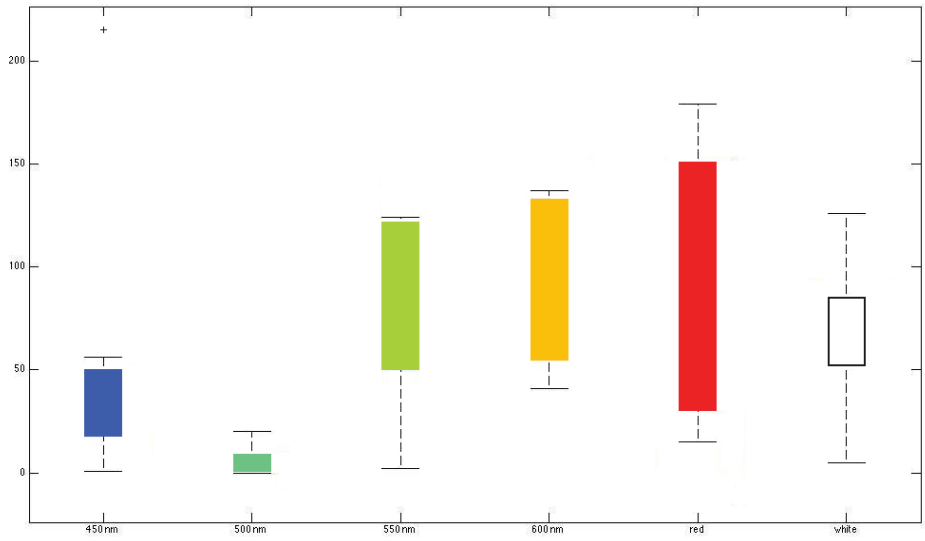


Fig. 9

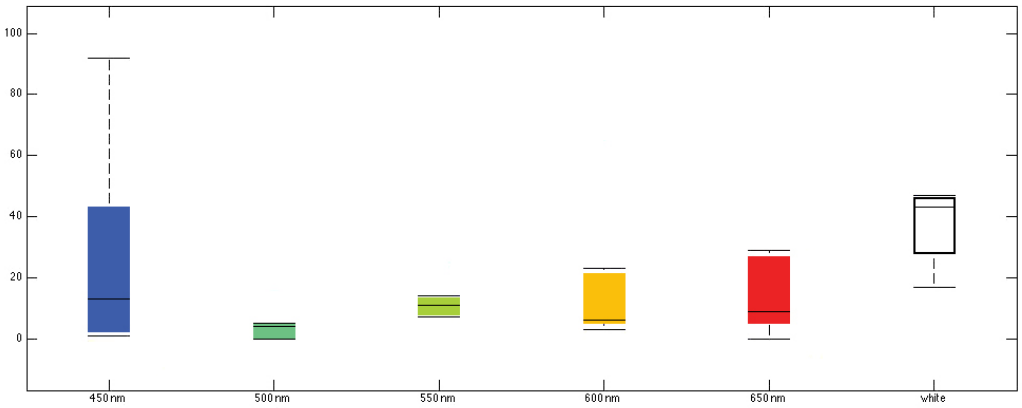


Fig. 10

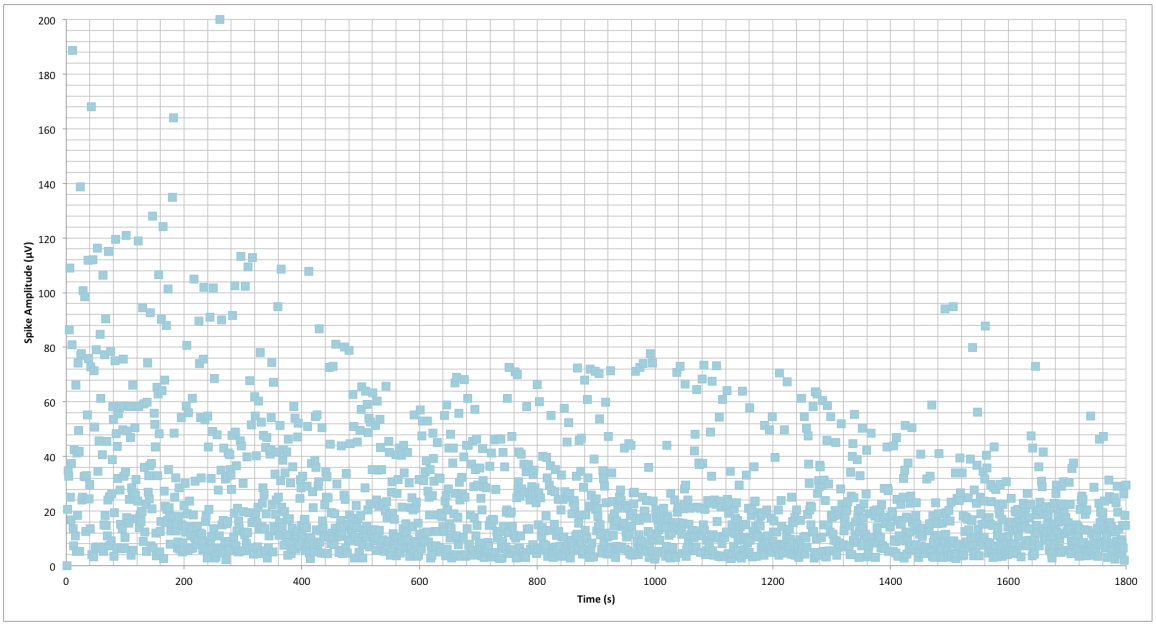
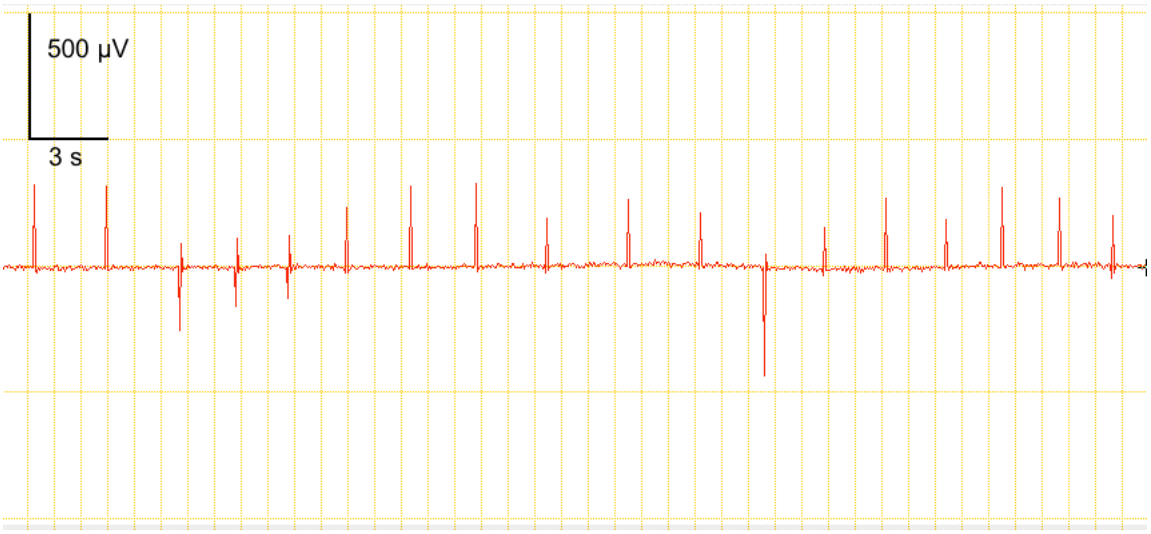
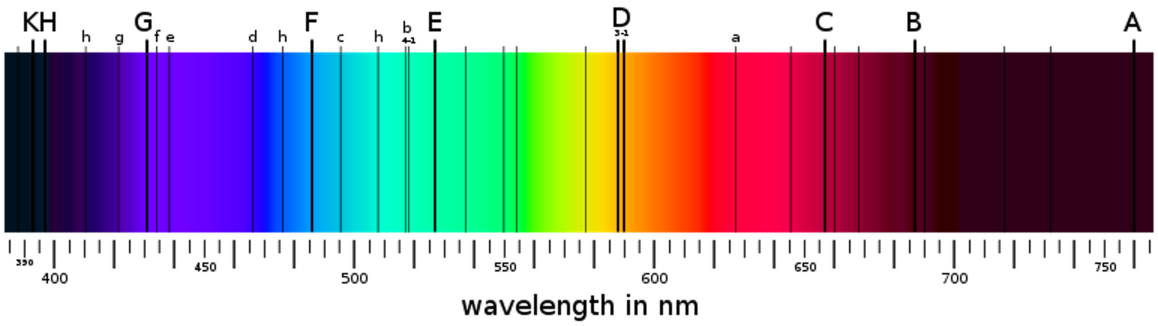

Spike Amplitude (μV)
200
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Time (s)

Fig. 11



Supplementary Fig. 1



CHAPTER 4

Effects of Light on Isolated Hypostomes of Hydra vulgaris

Stephanie L. Guertin[2], and Gabriele Kass-Simon[1,2], *

[1]Department of Biological Sciences, College of the Environmental and Life Sciences, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881-0816

[2]Interdisciplinary Neurosciences Program, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881-0816

* Author for correspondence (email: gkass.simon@uri.edu)

In preparation for submission to:

Comparative Biochemistry and Physiology A: Physiology

Abstract:

The early-evolving cnidarian *Hydra vulgaris* has a complicated behavioral and electrical response to light, as has been shown in a variety of previous work. Light changes both the large head pulses and bursts, and the small pulses and rhythmic potential system. Here, we recorded from the isolated hypostomes of hydra across a spectrum from 450 to 650 nm including red light, and using white light as a baseline. We found uniformly low levels of activity in all pulse types in the isolated hypostomes, activity that appeared to decrease with the application of light. Due to the extremely low levels of impulses, though, this decrease fell below our significance threshold. We hypothesize that the head serves primarily as an integration center for light signals, rather than a locus for their perception.

Introduction:

Hydra are early-evolved metazoans of the class Cnidaria, tubular coelenterates whose bodies consist of two epithelial layers, the endoderm and ectoderm, separated by an acellular middle layer, the mesoglea. A ring of five to eight tentacles surrounds a mouth located at the tip of the hypostome. The animals attach to the substrate via adhesion of a basal disc at the aboral end of the body column. Hydras possess a complete ectodermal nerve net and have gap-junctional connections between the ectodermal and endodermal epithelial cells (Hufnagel and Kass-Simon 1976, Wood 1979). Histological evidence indicates that there are two nerve rings in the hypostome, one of which appears to be coincident with the physiologically defined ring that integrates and coordinates tentacle and body contractions (Hufnagel & Kass-Simon 1976, Kass-Simon 1973, Kinnamon 1981, Koizumi 1992, Rushforth 1971, 1973, Passano and McCullough 1962, 1964, 1965).

Despite their relatively simple anatomical organization, hydra have a complex variety of sensory modalities, including mechanical and chemical (Kass-Simon and Scappaticci, 2002, Thurm 2004). Of particular interest is their response to light. Hydra, unlike some other cnidarians, bear no eyes, eyespots, or other distinct photoreceptive structures. This extraocular photosensitivity may be the oldest extant type of photosensitivity, appearing in a variety of species from bacteria through vertebrates, and present in non-specialized cells, neurons, and loose aggregations of photopigment in cells (Arendt 2003). Though lacking in specific photoreceptive structures, hydra do have a variety of specialized sensory and effector cells, some of which may play a role in photosensitivity. Candidates for this include sensory neurons

of the ectoderm, battery cells of the tentacle, and nematocytes in body and tentacle. Despite the responsible cell type remaining unknown, hydra have a well-developed light response that has been known since the 1890s (Wilson 1891). The most immediately obvious evidence of this is their frequently described 'somersaulting' behavior, which allows them to quickly move toward a light source (Ewer 1947, Feldman and Lenhoff, 1962)

In Wilson's early experiments, *Hydra pirardi* were exposed to different colors of light by means of panes of colored glass placed over their aquarium and quantified by the adjacent Fraunhofer lines (Wilson 1891). The Fraunhofer lines are a series of measured points corresponding to dark lines on the visible emission spectrum of the sun. The animals were found to congregate preferentially under blue light. Haug (1933) found that hydra appeared unresponsive to red light altogether, with pacemaker activity that did not differ from that in ambient light.

Later work by Passano and McCullough showed that shining white light on a narrow band at the foot of dark-adapted *Hydra vulgaris* caused first extension and then contraction (Passano and McCullough 1964). Additionally, in these whole animals, exposure to light of less than 500 nm produced a decrease in the frequency of contractions pulses of the endodermal musculature, resulting in body column extension, as well as an increase in the rate of rhythmic potentials. The contractile bursts were also susceptible to interruption by light, as a six-second burst of bright light would stop a current burst. Subsequent bursts would increase in number of pulses per burst after such an interruption, with the number of pulses per burst continuing to increase with every time that a burst was interrupted for a single animal.

In bodiless head and tentacle preparations, as well as headless bodies, exposure to pulses of colored light changed the frequency and timing of both ectodermal contraction pulses and endodermal rhythmic waves (Taddei-Ferretti et al. 2004). In addition Taddei-Ferretti, et al describe a large slow electrical change in baseline which the authors refer to as "the big slow wave" which does not seem to be related to the classical rhythmic pulses.

In other experiments, *H. pirardi* was shown to have the swiftest change in behavior patterns when exposed to 350 to 500 nanometer light, with responses decreasing markedly above 500 nm before returning slightly at above 575 nm. The contractile response was also affected by the intensity of the light, with the speed of the onset of the first contraction directly proportional to the intensity. Intensity of light was also shown to affect the tentacle contraction pulses in isolated tentacles, with pulses of strong white light causing an increase in contraction bursts compared to ambient light (Singer, 1963).

The results in light seen by Passano and McCullough, Taddei-Ferretti, et al., and Singer all give a picture of a very defined photoresponse, with the greatest increase in activity in high intensity light below a wavelength of 500 nm. Despite this clearly defined response, the anatomical diversity of the responding regions of the animal has made discernment of the specific cell types and pathways involved in the photoresponse unclear. Either this photosensitivity is found in many cells, or the cell types responsible are widely distributed.

A number of attempts to locate the genetic basis of this photosensitivity have also been made. Multiple opsin-like photopigment genes have been isolated from

hydra genomic DNA using degenerate primers formed from the rhodopsin genes in twelve different invertebrates; the finding of photopigments that respond to different wavelengths is consistent with the suggestion that hydra are capable of discerning colors (Santillo et al. 2006). Additionally, in hydra, one such opsin has been shown to colocalize with the expression of genes for cyclic nucleotide-gated (CNG) proteins, like those found in the phototransduction cascade in other taxa (Plachetzki et al. 2007). Inactivation of the CNG channels resulted in a loss of firing of nematocysts when the animal was exposed to light (Plachetzki et al. 2012). *Hm2* was particularly concentrated around the mouth, in a pattern that appears to mirror the locations of the proximal hypostomal nerve ring described by Hufnagel and Kass-Simon (Kass-Simon et al. 2007 and unpublished).

Because both the body region responsible for the reception of light and the specific neural circuitry involved remain unclear, we removed the tentacles and body, allowed the isolated heads to heal, and recorded extracellularly from the base of the head. This allowed for examination of the responses of the hypostome when isolated from the nerve network of the rest of the animal. Not only would this allow for examination of the responses of the cells of the hypostome to light, but a difference in response patterns between isolated hypostomes and isolated tentacles or whole bodies would indicate that processing is occurring after the reception of the light signal.

Materials and Methods:

Animal culture:

Specimens *of Hydra vulgaris* were asexually cultured in glass baking dishes containing modified bicarbonate versene culture solution (BVC): 1 x 10 - 7 mol/L NaHCO3, 1 x 10-6 mol/L CaCl2, and 1 x 10-8 mol/L EDTA, pH 7.0 +/- 0.2 (Loomis and Lenhoff 1956, Muscatine and Lenhoff 1965). All animals were kept in darkness in an incubator at 18 +/- 1.0 °C. Hydra cultures were fed freshly hatched *Artemia salina* nauplii once every 48 h.

Experimental Preparations:

Twenty-four hour starved animals were placed in BVC in a petri dish under a light microscope and allowed to relax. For hypostomal recordings, 24-hour starved animals were placed in BVC and allowed to relax. At maximal extension, the head was removed just below the point of tentacle insertion. The tentacles were then excised to produce a ring of hypostomal tissue free of the tentacular nervous system (Fig. 1). Care was taken to not damage the portions of the hypostome considered to contain the nerve ring by previous authors (Hufnagel 1985, Koizumi 1992). These tentacle-free heads were then allowed to heal for six hours before recording.

Electrophysiology:

A single head was attached to a suction electrode in BVC under a dissecting microscope. The experimental protocol was as follows: ten minutes of darkness, ten minutes of the tested light wavelength, ten minutes of white light. Colored light was

provided with the use of narrow bandpass (50 nm) filters at 450, 500, 550, and 600 nanometers (Edmund Optics). All wavelengths were adjusted to a constant intensity of 1100 lux using neutral density filters (Edmund Optics) and measured with a Luna-Pro lightmeter. All electrical recordings were made at 22.0 C +/- 2.0 C, in 5 mL dishes filled with 3 mL of BVC, in complete darkness. Impulses were fed into the head stage of an A-M Systems Model 3000 amplifier and then into an AD Instruments PowerLab. The digital output was recorded with LabChart 7 on a MacBook Pro. The protocol was essentially that of Kay and Kass-Simon, 2009 and Ruggeri, et al., 2004. Impulses, characterized by their shape and size, were then measured during each of the three periods and compared using FANOVAs. Several types and patterns of pulses were observed. Contraction impulses often arise in bursts of several pulses close together in time; the number of pulses per burst and the number of bursts per time were compared. A burst was defined as at least three pulses arising within 15 s of each other and followed by a period of silence.

Hypostomal Pulses

The hypostomal recordings had three types of pulses: large uncorrelated head pulses (LUHPs), medium uncorrelated head pulses (MUHPs), and small uncorrelated head pulses (SUHPs.) Large uncorrelated head pulses originated from hypostomal tissue and ranged between 571 and 800 μV in size. Medium uncorrelated head pulses (MUHPs) ranged between 570 and 301 μV in size, and small uncorrelated head pulses between 30 and 300 μV. In contrast to tentacle pulses and rhythmic potentials, these uncorrelated pulses were not visibly associated with any external behavior of the

animal. A subset of the SUHPs was deemed to be rhythmic potentials (RPs); these were similar in size and shape to the SUHPs, but occurred in a recognizably regular pattern before stopping again (Fig. 2).

Data Analysis:

For each of the seven heads used for each experimental condition, pulses were visually identified and sorted, and their amplitudes recorded. These data were then processed in Matlab and compared using FANOVAs via the Matlab *friedman* command, with an alpha level of 0.05 and the corresponding post-hoc test to determine which of the wavelengths produced impulses that were significantly different from baseline using *multcompare*.

Results:

Head Pulses

Comparing total spike activity in heads in light to that in darkness, there initially appeared to be a was a universal decrease in the rate of small, medium, and large uncorrelated head pulses in any color of light. It was not, however significant at a p value of 0.05. (See Table 1 and 2). When the electrical responses of the isolated heads were compared across colors, none of the three size groups of head pulses showed a significant difference from any other. These tentacle free heads also did not show a significant change in rhythmic potentials.

Bursting Activity

In the isolated hypostomes, the usual pattern of contraction bursts was not observed. Activity levels were uniformly lower than that in isolated tentacles, with even the large uncorrelated head pulses primarily occurring singly scattered across the length of the recording periods.

This is not to say that activity was not observed. The isolated heads did demonstrate the usual types of activity, with pulses as large as ± 1mV and as small as ± 3μV easily observable. In some preparations, bursts of pulses were observed.

Discussion:

Diverse previous authors have shown light to affect the bursting activity of hydra, in various preparations and various ways. In our hands, the isolated hypostome, after removal of body and tentacles, all colors of light appeared to result in a reduced spiking activity relative to darkness. However, no type of activity was found to be significantly affected by a change in the presence or color of light. It might be argued that the actual cause of the lack of differential response is due to the recording method - a primary side effect of extracellular recording in this manner is the uneven ability to pick up impulses from differently located responding cells. It might be possible, then, that sensory cells far from the site of the electrode are responding to the changes in light, but the pulses are not being through-conducted to the site of the electrode. In this case, however, that is not the cause - both large and small pulses were observed in our recordings. What was not observed was a differential response to colored light.

The lack of difference in response to colored light found here in our hypostomal preparations indicates that although the nerve rings in the hypostome may be necessary for the complicated bursting patterns and responses seen in whole animals, tentacles, and bodiless heads with tentacles, the hypostome alone is not sufficient for the full spectrum of photobehavior.

This argues for a primary role for the hypostome in integration of inputs from the tentacles and body and coordination of the eventual response, rather than direct sensory response.

Additionally, in these and other experiments (Lauro and Kass-Simon, unpublished), the level of bursting activity of the isolated and unstimulated

hypostome in darkness is universally low, if found at all. This is even truer when the activity levels in the isolated hypostome are compared to the isolated tentacles; the tentacles alone show both more frequent pulses and a variety of single pulse, burst, and train behaviors. In the hypostome, activity patterns more in keeping with previous research on whole animals or head and tentacle preparations, including bursts, will return when the hypostome is allowed to heal for a longer period and exposed to a chemical stimulus like reduced glutathione. It may be surmised from this, and from the immunohistochemical work of Hufnagel et al. on the tentacles and heads, that the cells responsible for the head contractile burst pacemaker activity are contained not only within the nerve ring itself, but up into the bases of the tentacles. As the tentacles regenerate, these pacemaker loci reform and regenerate.

The hypostome, then, serves as an organizational center for the pulses that originate, change, and are later conducted in and out of the tentacles and body.

References:

Arendt, D. 2003. Evolution of eyes and photoreceptor cell types. *Int. J. Dev. Biol*, 47: 563571.

Feldman, M, and HM Lenhoff. 1960. Phototaxis in Hydra littoralis: rate studies and localization of the "photoreceptor." *Anat. Rec*. 137: 354-355.

Haug, G. 1933. Die Lichtreaktionen der Hydren (Chlorohydra viridissima und Pelmatohydra oligactis). *Z. Vgl. Physiol*. 19: 246–303.

Hufnagel, L and G. Kass-Simon. The ultrastructural basis for the electrical coordination between epithelia of hydra. *Coelenterate Ecology and Behavior: Selected Papers* (1976).

Kass-Simon, G. 1970. Multiple excitation sites and straight-line conduction in the contraction burst system of Hydra. *Am. Zool. 10: 505*.

Kass-Simon, G. 1972. Longitudinal conduction and contraction burst pulses from hypostomal excitation loci in Hydra attenuata. *J. Comp. Physiol*. 80: 29–49.

Kass-Simon, G. 1973. Transmitting systems in Hydra. *Publ. Seto Mar. Biol. Lab*. 20: 583–593.

Kass-Simon, G, and L Passano. 1978. A neuropharmacological analysis of the pacemakers and conducting tissues of Hydra attenuata. *J. Comp. Physiol. A,* 128, 7179.

Kass-Simon, G, and P Pierobon. 2007. Cnidarian neurochemical transmission: an

updated overview. *Comp. Biochem. Physiol. A* 146: 9 –25.

Kass-Simon, G, and AA Scappaticci. 2002. The behavioral and developmental physiology of nematocysts. *Can. J. Zool.* 80: 1772– 1794.

Kinnamon, JC, and JA Westfall. 1981. A three dimensional serial reconstruction of neuronal distribution in the hypostome of Hydra. *J. Morphol.* 168: 321–329.

Koizumi, O, Itazawa, M, Mizumoto, H, Minobe, S, Javois, L, Grimmelikhuijzen, CJP, and HR Bode. 1992. Nerve ring of the hypostome in Hydra. I. Its structure and maintenance. *J. Comp. Neurol.* 326: 7–21.

Loomis, WF, and HM Lenhoff. 1956. Growth and sexual differentiation of *Hydra* in mass culture. *J. Exp. Zool.* 132: 555–573.

Muscatine, L, and HM Lenhoff. 1965. Symbiosis of hydra and algae. II. Effects of limited food and starvation on growth of symbiotic and aposymbiotic hydra. *Biol. Bull.* 129: 316–328.

Passano, LM, and CB McCullough. 1962. Light response and the rhythmic potentials of *Hydra*. *Proc. Natl. Acad. Sci. USA* 48: 1376– 1382.

Passano, LM, and CB McCullough. 1963. Pacemaker hierarchies controlling behavior of *Hydra*. *Nature* 199: 1174–1175.

Passano, LM, and CB McCullough. 1964. Coordinating systems and behavior in *Hydra*. I. Pacemaker system of the periodic contractions. *J. Exp. Biol.* 41: 643–644.

Passano, LM, and CB McCullough. 1965. Coordinating systems in *Hydra:* the rhythmic potential system. *J. Exp. Biol.* 42: 205–231.

Plachetzki, D, Degnan, B, and TH Oakley. 2007. The Origins of Novel Protein Interactions during Animal Opsin Evolution. *PLoS ONE*, 2 (10), e1054.

Plachetzki D, Fong CR, and TH Oakley. 2012. Cnidocyte discharge is regulated by light and opsin-mediated phototransduction. *BMC Biol.* 10:17.

Ruggieri, RD, Pierobon, P, and G Kass-Simon. 2004. Pacemaker activity in *Hydra* is modulated by glycine receptor ligands. *Comp. Biochem. Physiol. A* 138: 193–202.

Rushforth, NB, and DS Burke 1971. Behavioral and electrophysiological studies of *Hydra*. II. Pacemaker activity of isolated tentacles. *Biol. Bull.* 140: 502–519.

Singer RH, Rushforth NB, and AL Burnett. 1963. The photodynamic action of light on Hydra. *J Exp Zool.* 154:169–173.

Taddei-Ferretti C, Musio C, Santillo S, and A Cotugno. 2004. The photobiology of hydra's periodic activity. *Hydrobiologia*. 129–134.

Taddei-Ferretti C, and C Musio. 2000. Photobehavior of hydra (Cnidaria, Hydrozoa) and correlated mechanisms: a case of extraocular photosensitivity. *J Photochem Photobiol B, Biol.* 55:88–101.

Thurm U, Brinkmann M, Golz R, Holtmann M, and D Oliver. 2004. Mechanoreception and synaptic transmission of hydrozoan nematocytes. *Hydrobiologia*. 530/531:97-105.

Wilson, EB. 1891. The heliotropism of hydra. *Am. Nat.* 25: 413–433.

Fig. 1

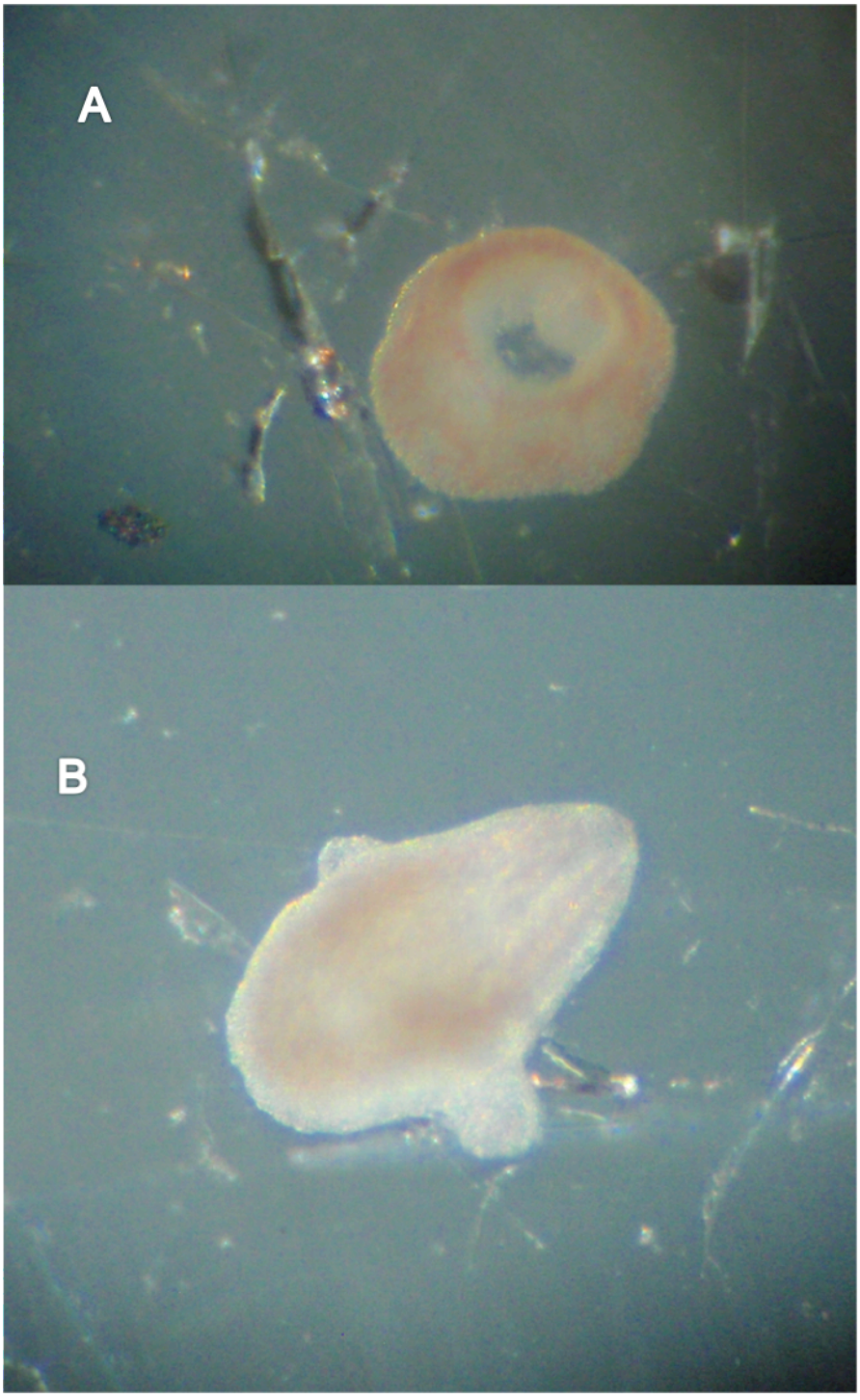


Fig. 2

Table 1

Head Pulses

Wavelength		Mean and SD	Median and IQR
450 nm: Blue (n=7)	SUHPs	2.43 ± 3.82	0 ± 3.5
	MUHPs	4.85 ± 6.84	0 ± 9.5
	LUHPs	0.71 ± 1.49	0 ± 0.5
500 nm: Green (n=7)	SUHPs	3.00 ± 3.87	1 ± 5
	MUHPs	4.71 ± 5.47	5 ± 6.5
	LUHPs	1 ± 1.53	0 ± 1.5
550 nm: Yellow (n=7)	SUHPs	3.00 ± 3.46	2 ± 5
	MUHPs	3.42 ± 3.99	2 ± 6
	LUHPs	0.43 ± 0.79	0 ± 0.5
600 nm: Orange (n=7)	SUHPs	12.14 ± 17.46	1 ± 17.5
	MUHPs	15.42 ± 21.90	2 ± 22.5
	LUHPs	1.42 ± 1.51	1 ± 3
650 nm: Red (n=7)	SUHPs	0.14 ± 0.37	0 ± 0
	MUHPs	0.28 ± 0.75	0 ± 0
	LUHPs	0.14 ± 0.38	0 ± 0
White (n=7)	SUHPs	3.00 ± 5.91	0 ± 2.5
	MUHPs	3.28 ± 6.24	0 ± 3
	LUHPs	0.28 ± 0.48	0 ± 0.5

Table 2

Head Pulses Change from Darkness

Wavelength		Mean and SD	Median and IQR
450 nm: Blue (n=7)	SUHPs	-1.43 ± 4.99	-2 ± 7.5
	MUHPs	-6.28 ±10.06	-5 ± 10
	LUHPs	-2.43 ± 4.19	-3 ± 6.5
500 nm: Green (n=7)	SUHPs	-4.14 ± 7.71	-1 ± 10.5
	MUHPs	-5.71 ± 10.77	-3 ±14
	LUHPs	-1.71 ± 4.57	0 ± 7
550 nm: Yellow (n=7)	SUHPs	-3.14 ± 2.11	-3 ± 2.5
	MUHPs	-4.00 ± 3.51	-4 ± 2.5
	LUHPs	0.43 ± 0.79	0 ± 0.5
600 nm: Orange (n=7)	SUHPs	-1.43 ± 14.59	-5 ± 5
	MUHPs	-0.85 ± 14.03	-6 ± 18.5
	LUHPs	-2.28 ± 4.86	-1 ± 5.5
650 nm: Red (n=7)	SUHPs	-4.14 ± 3.53	-3 ± 5.5
	MUHPs	-8.00 ± 6.71	-7 ± 10
	LUHPs	-2.42 ± 3.05	-1 ± 1.5
White (n=7)	SUHPs	-7.43 ± 12.79	-6 ± 10.5
	MUHPs	-12.00 ± 16.50	-9 ± 12.5
	LUHPs	0.57 ± 4.08	0 ± 1.5